Engineering *Escherichia coli* for methanol conversion

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ABSTRACT

Methylotrophic bacteria utilize methanol and other reduced one-carbon compounds as their sole source of carbon and energy. For this purpose, these bacteria evolved a number of specialized enzymes and pathways. Here, we used a synthetic biology approach to select and introduce a set of "methylotrophy genes" into *Escherichia coli* based on *in silico* considerations and flux balance analysis to enable methanol dissimilation and assimilation. We determined that the most promising approach allowing the utilization of methanol was the implementation of NAD-dependent methanol dehydrogenase and the establishment of the ribulose monophosphate cycle by expressing the genes for hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexuloisomerase (Phi). To test for the best-performing enzymes in the heterologous host, a number of enzyme candidates from different donor organisms were selected and systematically analyzed for their *in vitro* and *in vivo* activities in *E. coli*. Among these, Mdh2, Hps and Phi originating from *Bacillus methanolicus* were found to be the most effective. Labeling experiments using ^{13}C methanol with *E. coli* producing these enzymes showed up to 40% incorporation of methanol into central metabolites. The presence of the endogenous glutathione-dependent formaldehyde oxidation pathway of *E. coli* did not adversely affect the methanol conversion rate. Taken together, the results of this study represent a major advancement towards establishing synthetic methylotrophs by gene transfer.

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1. Introduction

Methylotrophy represents the ability of microorganisms to utilize reduced one-carbon (C_1) compounds such as methanol or methane as their sole source of carbon and energy. Substantial knowledge in the field has been generated in the past 50 years in particular regarding the occurrence of this trait in microorganisms as well as the set of enzymes and pathways required for methylotrophic growth. Methanol is a non-food alternative substrate to sugar for microbial bioprocess with a competitive price and a sustainable supply. In consequence, interest in methylotrophy is fueled by biotechnological interest in converting raw materials alternative to sugars by microbial fermentation. Although methanol was applied for industrial-scale production of

single-cell proteins in the 1970s (Maclenna et al., 1973; Solomons, 1983; Westlake, 1986; Windass et al., 1980), in the past decades interest has shifted to transforming methanol into value-added products including bulk chemicals such as biofuels, amino acids and biopolymers (Schrader et al., 2009; Brautaset et al., 2007), and a methanol-based economy as an alternative fuel and feedstock concept has been proposed (Olah, 2013).

A key question to understanding methylotrophy is how methylotrophic organisms generate energy and how they convert C_1 substrates such as methanol into carbon compounds with carbon-carbon bonds (carbon fixation and biomass formation). Research efforts with different model species have revealed that methylotrophy consists of a set of functional modules that are ultimately linked to central metabolism and genomes from well-studied model methylotrophs have been determined (Heggeset et al., 2012; Irla et al., 2014; Marx et al., 2012; Vuilleumier et al., 2009). In all methylotrophs, methanol metabolisation is initiated by its oxidation to formaldehyde either by a periplasmic

pyrroloquinoline quinone (PQQ)-containing methanol dehydrogenase in Proteobacteria or an NAD-linked cytoplasmic enzyme in Gram-positives. Because formaldehyde represents an essential but cell-toxic intermediate, its conversion must be efficient. On the one hand, formaldehyde is converted for energy generation *via* non-orthologous pathways (*i.e.*, the ribulose monophosphate (RuMP) pathway, the tetrahydromethanopterin-linked pathway, the glutathione-linked pathway). On the other hand, C_1 assimilation for biomass formation occurs with or without net CO_2 fixation (RuMP cycle, serine cycle, Calvin cycle) (Anthony, 1982; Chistoserdova, 2011; Chistoserdova and Lidstrom, 2013; Vorholt, 2002).

Although it will remain crucial to continue efforts to better understand methylotrophy in natural methylotrophs by using them as the object of interest, we here launch a parallel strategy using synthetic biology concepts related to metabolic engineering with the long term goal of converting non-methylotrophs into methylotrophs. This approach aims at (i) increasing our fundamental understanding of the process, and (ii) providing access to methanol as a raw material using established platform organisms for methanol conversion into value-added products. The value of applying concepts of design strategies has been noted and critically reviewed (*e.g.*, (Way et al., 2014)) and great progress has been made in providing new standardized working tools and successfully engineering bacterial metabolism for the production of chemicals. Success in implementing new pathways to "drain" intermediates of central metabolism has been reported for the synthesis of value-added products such as artemisinin and biofuels (Kung et al., 2012; Ro et al., 2008). Such product formation had been achieved from carbohydrates (*e.g.* pentoses and glycerol), but hitherto not from C_1 substrates.

Implementing new pathways for carbon influx from a C_1 compound represents a difficult task because of the challenging condensation of C_1 compounds to central metabolites. So far, attempts have been initiated to introduce carbon fixation pathways, and in one study all genes for enzymes for the 3-hydroxypropionate bicycle have been expressed (Mattozzi et al., 2013); however, to our knowledge, no autotrophic pathway has yet been successfully introduced that sustains biomass formation. Additionally, a synthetic CO_2-fixing photorespiratory bypass has been implemented recently (Shih et al., 2014). The aim of generating a synthetic methylotroph represents a particular challenge because both energy demands and biomass requirements must be met by a C_1 source. Moreover, as mentioned above, methylotrophy involves a toxic intermediate and thus any imbalance in metabolism might have fatal consequences.

Here, we use the model bacterium *Escherichia coli* to implement enzymes and pathways for methanol conversion. This choice is justified by the extensive knowledge about its metabolism including a stoichiometric metabolic model (Feist et al., 2007) and the biotechnological interest in the bacterium. Our strategy involves *in silico* modeling and rational criteria for enzyme selection followed by expression of the genes from various donor organisms for empirical testing of their suitability in cell extracts and *in vivo*. We demonstrate that pathways can be successfully established as evidenced by multiple labeled sugar intermediates up to fully labeled hexoses from methanol (C_1).

2. Material and methods

2.1. In silico modeling

For *in silico* modeling approaches the Optflux software (Rocha et al., 2010) version 3.0.7 was used. For modeling we used an *E. coli* model based on (Feist et al., 2007) and added reactions of an NAD-dependent Mdh, a PQQ-dependent Mdh and its associated cytochrome *c* oxidase, Hps, Phi, serine glyoxylate aminotransferase,

malate thiokinase and malyl-CoA lyase. Furthermore, a reaction was added allowing the creation of methylene-THF from formaldehyde. During project creation all external metabolites were removed. As biomass reaction R_Ec_biomass_iAF1260_core_59p81M was set.

2.2. Plasmid construction

Oligonucleotides used for construction were obtained from Microsynth (Switzerland) and are also listed in Supplementary Table 1. The plasmids constructed and used in this study are listed in Supplementary Table 2. DNA manipulations were performed using standard protocols (Green et al., 2012). Genes for methanol dehydrogenases and *act* from *Bacillus methanolicus* MGA3 and *B. methanolicus* PB1 were amplified from pET21a plasmids (Krog et al., 2013); other genes for alcohol dehydrogenases, hexulose-6-phosphate synthases and phospho-hexuloisomerases genes used in this study were amplified from genomic DNA. The artificial fusion of Hps and Phi and creation of Mdh activation mutant enzymes was achieved by overlapping PCR. NADP-sensitive Mdh mutant enzymes were constructed following the quick change protocol (Cormack and Castano, 2002). All PCR reactions were performed using Phusion polymerase (Finnzymes), and restriction enzymes were purchased from Fermentas. All constructs were verified by sequencing (Microsynth, Switzerland).

2.3. Gene expression in E. coli and preparation of cell free extracts

LB or M9 medium (400 mL) was inoculated to an OD600 of 0.1 using an overnight culture grown in the same medium. Cells grew to an OD600 of 0.4–0.6, gene expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Biosynth) and the cells were incubated at 37 °C, 150 rpm for five hours. The cultures were harvested by centrifugation (JA-10 rotor, Avanti J-E, Beckman Coulter) for 10 min at 8000g and 4 °C.

Cell free extract was prepared by resuspending cell pellets in 1 mL 50 mM dipotassium phosphate buffer (pH 7.4) supplemented with complete protease inhibitor cocktail tablets (cOmplete, EDTA-free, Roche Applied Science). The cells were disrupted by passing the cell suspension three times through a French pressure cell press (SLM instruments, Thermo Fisher Scientific) at 1000 Psi. Cell debris and membrane fractions were removed by ultracentrifugation (140,000g for 60 min at 4 °C). The resulting supernatant was used in enzyme assays.

2.4. Enzyme assays using cell free extracts

Assay of NAD-dependent methanol dehydrogenase activity in cell free extracts. The assay was performed in a total volume of 1 mL in pre-heated (37 °C) 50 mM K_2HPO_4 buffer supplemented with 5 mM $MgSO_4$. The standard assay contained 500 μM NAD^+ (Sigma-Aldrich) and 5–10 μL cell free extract corresponding to 150–300 μg total protein. The reaction was started by addition of 1 M methanol. The production of NADH was continuously measured at 340 nm at 37 °C with a Cary 50 Bio UV–visible spectrophotometer (Varian, Steinhausen, Switzerland). Activity was calculated from maximal slopes using the law of Lambert–Beer ($\varepsilon_{NADH} = 6220$ M^{-1} cm^{-1}). One unit (U) was defined as the amount of enzyme that is required to process 1 μmol of substrate per minute. If specific units (U/mg protein) are calculated, the total protein concentration of the lysate was used. Protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin (BSA) (Sigma-Aldrich, Buchs, Switzerland) as a standard. All measurements were performed at least in duplicates.

Hexulose-6-phosphate synthase activity assay. The activity of 3-hexulose-6-phosphate synthase (Hps) was determined in a discontinuous assay based on the formation of 3-hexulose 6-phosphate from