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Expression of heterologous non-oxidative pentose phosphate pathway from *Bacillus methanolicus* and phosphoglucose isomerase deletion improves methanol assimilation and metabolite production by a synthetic *Escherichia coli* methylotroph

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ABSTRACT

Synthetic methylotrophy aims to develop non-native methylotrophic microorganisms to utilize methane or methanol to produce chemicals and biofuels. We report two complimentary strategies to further engineer a previously engineered methylotrophic *E. coli* strain for improved methanol utilization. First, we demonstrate improved methanol assimilation in the presence of small amounts of yeast extract by expressing the non-oxidative pentose phosphate pathway (PPP) from *Bacillus methanolicus*. Second, we demonstrate improved co-utilization of methanol and glucose by deleting the phosphoglucose isomerase gene (*pgi*), which rerouted glucose carbon flux through the oxidative PPP. Both strategies led to significant improvements in methanol assimilation as determined by ^{13}C-labeling in intracellular metabolites. Introduction of an acetone-formation pathway in the *pgi*-deficient methylotrophic *E. coli* strain led to improved methanol utilization and acetone titers during glucose fed-batch fermentation.

1. Introduction

Abundant natural gas supplies in recent years have prompted considerable interest in using methane, the main component of natural gas, or its oxidized product, methanol, as substrates for biological production of fuels and chemicals (Haynes and Gonzalez, 2014). Since these one carbon (C1) compounds are more energy rich, i.e. possess a higher degree of reduction, than lignocellulosic sugars, their use is expected to enhance product titers and/or yields when used alone or in combination with sugars during fermentative processes compared to those using sugars alone (Gonzalez and Antoniewicz, 2017; Whitaker et al., 2015). As native methylotrophs have limited genetic tools, which are not as well-developed and extensive as those of platform organisms, and are mostly obligate aerobes, synthetic methylotrophy using platform organisms is of considerable interest for biological production of chemicals and biofuels (Whitaker et al., 2015). Several efforts have been made to achieve this in model organisms such as *Escherichia coli*, *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* (Dai et al., 2017; Lessmeier et al., 2015; Muller et al., 2015; Whitaker et al., 2017; Witthoff et al., 2015). We have recently reported the development of a methylotrophic *E. coli* strain that can grow on and convert methanol into the valuable chemical naringenin with a small amount of yeast extract supplementation. That strain was developed using episomal expression of a methanol dehydrogenase (MDH) gene (*mdh*) from *Bacillus stearothermophilus* and the ribulose monophosphate (RuMP) pathway, consisting of the hexulose phosphate synthase (*hps*) and phosphohexulose isomerase (*phi*) genes from *B. methanolicus* in a formaldehyde detoxification (*frmA*) deficient background (Fig. 1A) (Whitaker et al., 2017). In this study, we employ two novel metabolic engineering strategies, each of which enhanced methanol assimilation when compared to the previously reported parent (control) strain.

The first strategy was inspired by a native Gram-positive methylotroph, *B. methanolicus*. Methylotrophy in this prokaryote has been shown to be plasmid dependent (Brautaset et al., 2004). The native plasmid, pBM19, contains five genes (ribulose phosphate epimerase (*rpe*), fructose-bisphosphate aldolase (*fba*), sedoheptulose bisphosphate (*glpX*), phosphofructokinase (*pfk*) and transketolase (*tkt*)) coding for enzymes of the non-oxidative pentose phosphate pathway (PPP) in

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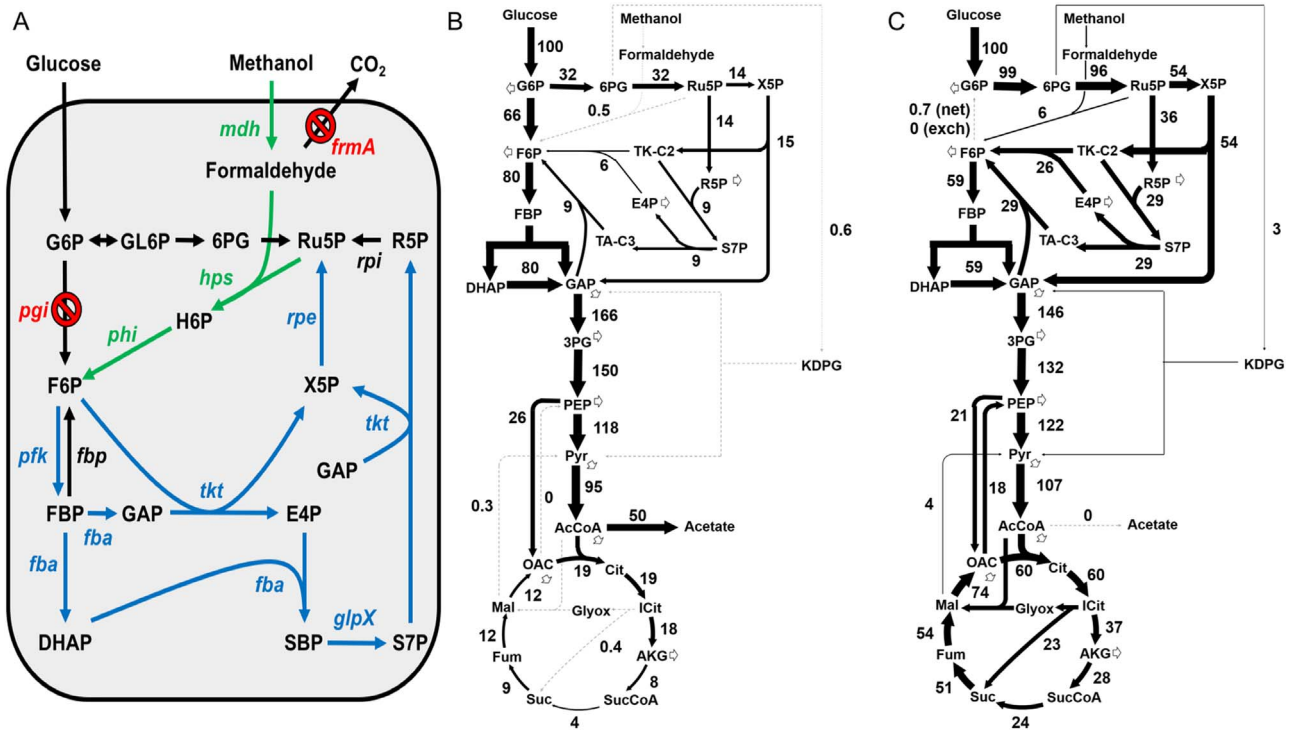

Fig. 1. Strategies to enhance synthetic methylotrophy. (A): Methanol is oxidized by the *mdh*-encoded MDH to formaldehyde, which is assimilated via the ribulose monophosphate (RuMP) pathway (*hps*, *phi*) to F6P upon reacting with Ru5P (green). Deletion of *frmA* eliminates formaldehyde dissimilation to CO_2 to conserve methanol carbon. The heterologous non-oxidative pentose phosphate pathway (PPP) from *Bacillus methanolicus* (*rpe*, *fba*, *glpX*, *pfk*, *tkt*) generates Ru5P from F6P (blue). Deletion of *pgi* reroutes glucose catabolism through the oxidative PPP to generate Ru5P from G6P. (B, C): Glucose carbon flux in methylotrophic *E. coli* Δ*frmA* (B) and methylotrophic *E. coli* Δ*frmA*Δ*pgi* (C). Enzymes (genes): methanol dehydrogenase (*mdh*), hexulose phosphate synthase (*hps*), phosphohexulose isomerase (*phi*), phosphoglucose isomerase (*pgi*), formaldehyde dehydrogenase (*frmA*), phosphofructokinase (*pfk*), fructose-bisphosphate aldolase (*fba*), transketolase (*tkt*), ribulose phosphate epimerase (*rpe*), sedoheptulose bisphosphate (*glpX*), fructose bisphosphatase (*fbp*), ribose phosphate isomerase (*rpi*). Metabolites: glucose 6-phosphate (G6P), 6-phosphogluconolactone (GL6P), 6-phosphogluconate (6PG), ribulose 5-phosphate (Ru5P), ribose 5-phosphate (R5P), hexulose 6-phosphate (H6P), fructose 6-phosphate (F6P), fructose bisphosphate (FBP), dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate (GAP), xylulose 5-phosphate (X5P), erythrose 4-phosphate (E4P), sedoheptulose bisphosphate (SBP), sedoheptulose 7-phosphate (S7P). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

addition to one copy of a methanol dehydrogenase gene (*mdh*) among other open reading frames that code for hypothetical proteins. Upon curing *B. methanolicus* of pBM19, methylotrophic capabilities were lost, i.e. growth on methanol was no longer possible, even when the *mdh* was expressed from a non-native plasmid. It was thus suggested that pBM19-borne genes, possibly the five non-oxidative PPP genes, are essential for growth on methanol in *B. methanolicus*. As part of the non-oxidative PPP, these enzymes are involved in the reversible conversion of fructose 6-phosphate (F6P) to ribulose 5-phosphate (Ru5P) (Fig. 1A). Since Ru5P is a critical intermediate for formaldehyde assimilation via the RuMP pathway, its generation is essential for sustaining methanol assimilation, which is first oxidized to formaldehyde via the MDH (Jakobsen et al., 2006). Thus, the heterologous non-oxidative PPP from *B. methanolicus* was integrated into the chromosome of the parent methylotrophic *E. coli* Δ*frmA* strain for recombinant expression (Fig. 2D). We demonstrate that the resulting strain exhibits improved methanol assimilation during growth in minimal media supplemented with a small amount of yeast extract.

The second strategy aimed at improving the co-utilization of a native substrate, notably glucose, with methanol, in the parent methylotrophic *E. coli* Δ*frmA* strain. As is, this strain catabolizes glucose primarily using glycolysis (Fig. 1B), which limits the amount of carbon flux through the PPP, likely limiting Ru5P generation for methanol assimilation (Long et al., 2016b; Toya et al., 2010; Usui et al., 2012). To overcome this potential limitation, a phosphoglucose isomerase (*pgi*) deficient methylotrophic *E. coli* strain was constructed (Fig. 1A). This strain, methylotrophic *E. coli* Δ*frmA*Δ*pgi*, must now catabolize glucose through the oxidative PPP, which greatly increases the amount of carbon flux toward Ru5P for improved methanol assimilation (Fig. 1C). We demonstrate that the *pgi*-deficient methylotrophic *E. coli* strain exhibits improved methanol assimilation, and thus co-utilization, during growth on glucose. To demonstrate the efficacy of the improved methylotrophic strain as applied to metabolite production, we engineered the *pgi*-deficient methylotrophic *E. coli* strain for acetone production from methanol and glucose. To accomplish this, we introduced the acetone-formation pathway from *Clostridium acetobutylicum*, consisting of the thiolase (*thl*), coenzyme A transferase (*ctfAB*) and acetoacetate decarboxylase (*adc*) (Fig. 5A) (Bermejo et al., 1998; Mermelstein et al., 1993). During glucose fed-batch fermentation, methanol utilization was improved in the methylotrophic *E. coli* Δ*frmA*Δ*pgi* strain compared to the parent methylotrophic *E. coli* Δ*frmA* strain. Additionally, acetone titers were improved in the methylotrophic *E. coli* Δ*frmA*Δ*pgi* strain when methanol was used as a co-substrate compared to the control culture without methanol. Taken together, this work details metabolic engineering approaches to enhance synthetic methanol assimilation in methylotrophic *E. coli* by targeting an essential RuMP pathway intermediate, Ru5P.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. ^{13}C-methanol (99% ^{13}C), [1,2-^{13}C]glucose (99.8%), and [1,6-^{13}C]glucose (99.2%) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). The isotopic purity and enrichment of glucose tracers was validated by GC-MS analysis (Antoniewicz et al., 2011). *E. coli* NEB5α, Q5 DNA polymerase and