Bioresources and Bioprocessing

SHORT REPORT

Biological conversion of methanol by evolved *Escherichia coli* carrying a linear methanol assimilation pathway

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Abstract

Background: Methanol is regarded as a biorenewable platform feedstock because nearly all bioresources can be converted into methanol through syngas. Biological conversion of methanol using synthetic methylotrophs has thus gained worldwide attention.

Results: Herein, to endow *Escherichia coli* with the ability to utilize methanol, an artificial linear methanol assimilation pathway was assembled in vivo for the first time. Distinct from native cyclic methanol utilization pathways, such as ribulose monophosphate cycle, the linear pathway requires no formaldehyde acceptor and only consists of two enzymatic reactions: oxidation of methanol into formaldehyde by methanol dehydrogenase and carboligation of formaldehyde into dihydroxyacetone by formolase. After pathway engineering, genome replication engineering assisted continuous evolution was applied to improve methanol utilization. ^{13}C-methanol-labeling experiments showed that the engineered and evolved *E. coli* assimilated methanol into biomass.

Conclusions: This study demonstrates the usability of the linear methanol assimilation pathway in bioconversion of C1 resources such as methanol and methane.

Keywords: Methanol, C1 resource, Synthetic methylotroph, *Escherichia coli*

Background

Methanol is a C1 compound that can be synthesized either from petrochemical or renewable resources (Carvalho et al. 2017; Patel et al. 2016). Owning to its cost advantage and biocompatibility, methanol is regarded as an attractive feedstock for production of biochemicals and biofuels (Pfeifenschneider et al. 2017). Although native methylotrophs are capable of using C1 resources including methanol as carbon and energy sources, they are more challenging to engineer than genetically tractable hosts due to inefficient genetic-transfer systems and editing tools (Whitaker et al. 2015).

Recently, synthetic methylotrophs were constructed by introducing native methanol assimilation pathways into non-native methylotrophs such as *Escherichia coli* (Dai et al. 2017; Leßmeier et al. 2015; Müller et al. 2015; Rohl-hill et al. 2017; Whitaker et al. 2017; Witthoff et al. 2015). To date, ribulose monophosphate (RuMP) cycle that utilizes ribulose-5-phosphate (Ru5P) as a formaldehyde acceptor is the only pathway used for synthetic methylotrophs. Despite the fact that Ru5P could be regenerated through pentose phosphate (PP) pathway, high coordination of heterologous RuMP cycle and native PP pathway is challenging (Whitaker et al. 2015). A computationally designed enzyme formolase (FLS) that can catalyze the carboligation of three formaldehyde molecules into one dihydroxyacetone (DHA) molecule was reported recently and used to construct an artificial carbon fixation pathway in vitro (Siegel et al. 2015). In the present study, an artificial linear methanol assimilation pathway based

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on FLS was assembled in *E. coli*, and a combined strategy of metabolic engineering and adaptive evolution was applied to facilitate methanol utilization.

Methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Additional file 1: Table S1. Gene expression and methanol utilization were performed in strain *E. coli* BW25113 Δ*frmA* (Ec-Δ*frmA*). The pTrc99A vector with a trc promoter was used for expression of the genes required for methanol utilization. Primers used for plasmid construction are listed in Additional file 1: Table S2. *E. coli* strains were cultured aerobically in lysogeny broth (LB) medium or M9 minimal medium supplemented with carbon sources at 30 or 37 °C. Detailed methods are described in Additional file 1: Additional methods.

Enzyme activity assay

Methanol dehydrogenase (MDH) and FLS activities were assayed using the methods described previously (Müller et al. 2015; Siegel et al. 2015). Detailed methods are described in Additional file 1: Additional methods.

Adaptive evolution

Detailed methods are described in Additional file 1: Additional methods.

Analysis of ^{13}C-labeling of proteinogenic amino acids

[13]C-labeling of proteinogenic amino acids was analyzed using a method described previously with some

modifications (You et al. 2012). Detailed methods are described in Additional file 1: Additional methods.

Results and discussion

Assembly of linear methanol utilization pathway in *E. coli*

The linear methanol utilization pathway consists of two steps: oxidation of methanol into formaldehyde and carboligation of formaldehyde into DHA, which can be phosphorylated to dihydroxyacetone phosphate by dihydroxyacetone kinase and enter lower glycolysis (Fig. 1a). According to the calculation by eQuilibrator (Flamholz et al. 2012), the $\Delta_r G'^o$ values for the linear pathway and the RuMP pathway are 9.1 and − 3.4 kJ/mol, respectively, suggesting that the RuMP pathway is more thermodynamically feasible. However, the product of the linear pathway can enter glycolysis and be metabolized quickly, providing a strong driven force for methanol utilization. Therefore, the linear pathway is also supposed to be feasible. To assemble the linear pathway in vivo, NAD⁺-dependent MDH from *Bacillus methanolicus* and artificial FLS were overexpressed in *E. coli*. The multicopy plasmid pTrc99A with a strong trc promoter was used to achieve high-level expression of MDH and FLS since their specific activities are quite low (Krog et al. 2013; Siegel et al. 2015). Two recombinants Ec-Δ*frmA*-*mdh3*$_{MGA3}$-*fls* and Ec-Δ*frmA*-*mdh2*$_{PB1}$-*fls* carrying the *fls* gene and different *mdh* genes (Additional file 1: Table S3) were constructed. Enzyme activity assays demonstrated that both MDHs were functionally expressed. Strain Ec-Δ*frmA*-*mdh2*$_{PB1}$-*fls* showed higher methanol oxidation activity that was approximately twice as high as the
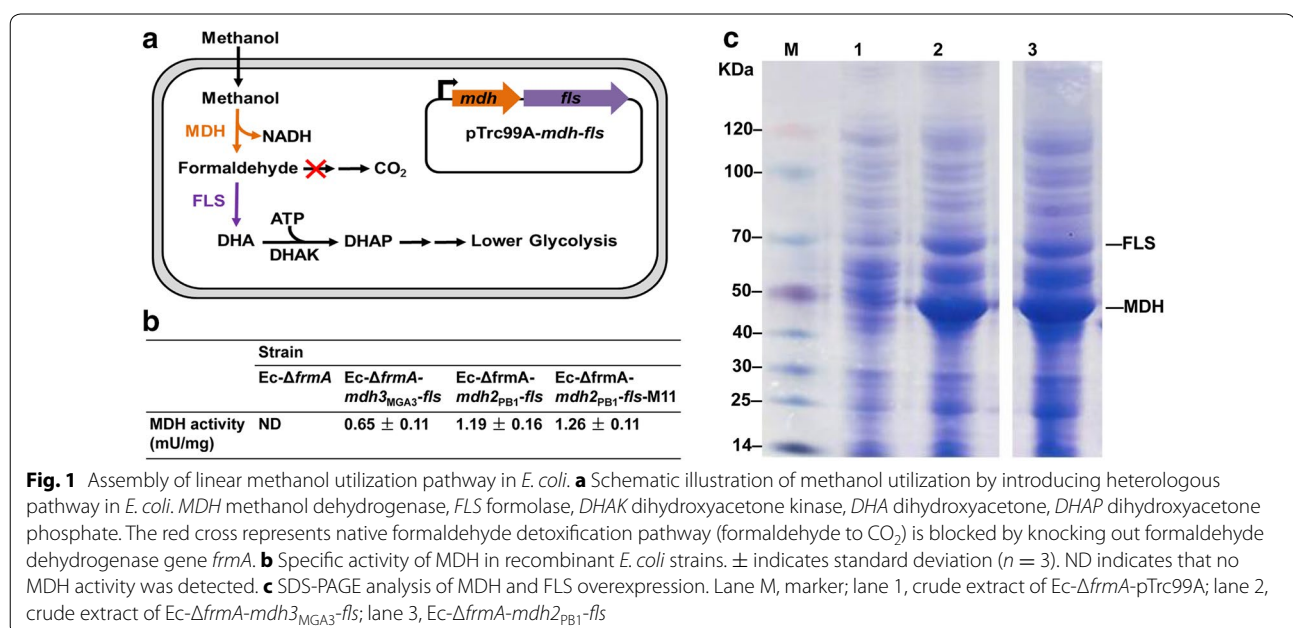

Fig. 1 Assembly of linear methanol utilization pathway in *E. coli*. **a** Schematic illustration of methanol utilization by introducing heterologous pathway in *E. coli*. *MDH* methanol dehydrogenase, *FLS* formolase, *DHAK* dihydroxyacetone kinase, *DHA* dihydroxyacetone, *DHAP* dihydroxyacetone phosphate. The red cross represents native formaldehyde detoxification pathway (formaldehyde to CO_2) is blocked by knocking out formaldehyde dehydrogenase gene *frmA*. **b** Specific activity of MDH in recombinant *E. coli* strains. ± indicates standard deviation ($n = 3$). ND indicates that no MDH activity was detected. **c** SDS-PAGE analysis of MDH and FLS overexpression. Lane M, marker; lane 1, crude extract of Ec-Δ*frmA*-pTrc99A; lane 2, crude extract of Ec-Δ*frmA*-*mdh3*$_{MGA3}$-*fls*; lane 3, Ec-Δ*frmA*-*mdh2*$_{PB1}$-*fls*

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MDH activity of strain Ec-Δ*frmA-mdh3*_{MGA3}-*fls* (Fig. 1b). FLS activity was determined by coupled reactions involving DHA formation from formaldehyde by FLS and DHA reduction by NAD^+-dependent glycerol dehydrogenase. However, the reverse activity of MDH that could reduce formaldehyde to methanol with NADH consumption interfered with the NADH-dependent DHA reduction. Therefore, FLS activity was not determined here, whereas SDS-PAGE analysis indicated that MDHs and FLS were successfully expressed (Fig. 1c). Strain Ec-Δ*frmA-mdh2*_{PB1}-*fls* was used in the subsequent experiments for higher MDH activity.

Bioconversion of methanol into biomass of the engineered *E. coli*

Despite the equipment of linear methanol assimilation pathway, the engineered strain could not initiate growth in M9 minimal medium with methanol (approximately 8 g/L, 1% v/v) as the sole carbon source. Similar phenomena were observed in previous studies on RuMP-based synthetic methylotrophs and undefined supplements such as yeast extract and tryptone were added to initiate cell growth on methanol (Whitaker et al. 2017). Thus,

small amounts of yeast extract (1 g/L) were added in M9 minimal medium. Any improvements in cell growth in the presence of methanol might derive from the contribution of methanol assimilation. As controls, a Δ*frmA* strain containing the empty pTrc99A vector (strain Ec-Δ*frmA*-pTrc99A) was cultivated using the aforementioned media. A methanol evaporation control without inoculation was also conducted.

As shown in Fig. 2a, approximately 1 g/L methanol evaporated away during the cultivation. When the control strain Ec-Δ*frmA*-pTrc99A was cultivated using yeast extract and methanol as co-substrate, no additional methanol consumption but slightly decrease in cell growth was observed (Fig. 2a, b). We speculated that methanol might be oxidized to toxic intermediate formaldehyde by the non-specific activities of alcohol dehydrogenases of *E. coli*, which affected the cell growth negatively. Regarding to strain Ec-Δ*frmA-mdh2*_{PB1}-*fls*, no significant increase in biomass was observed when methanol was added (Fig. 2c), whereas slightly more methanol (1.45 g/L) was consumed compared to the evaporation control, suggesting methanol utilization of the engineered strain. To further validate methanol utilization,
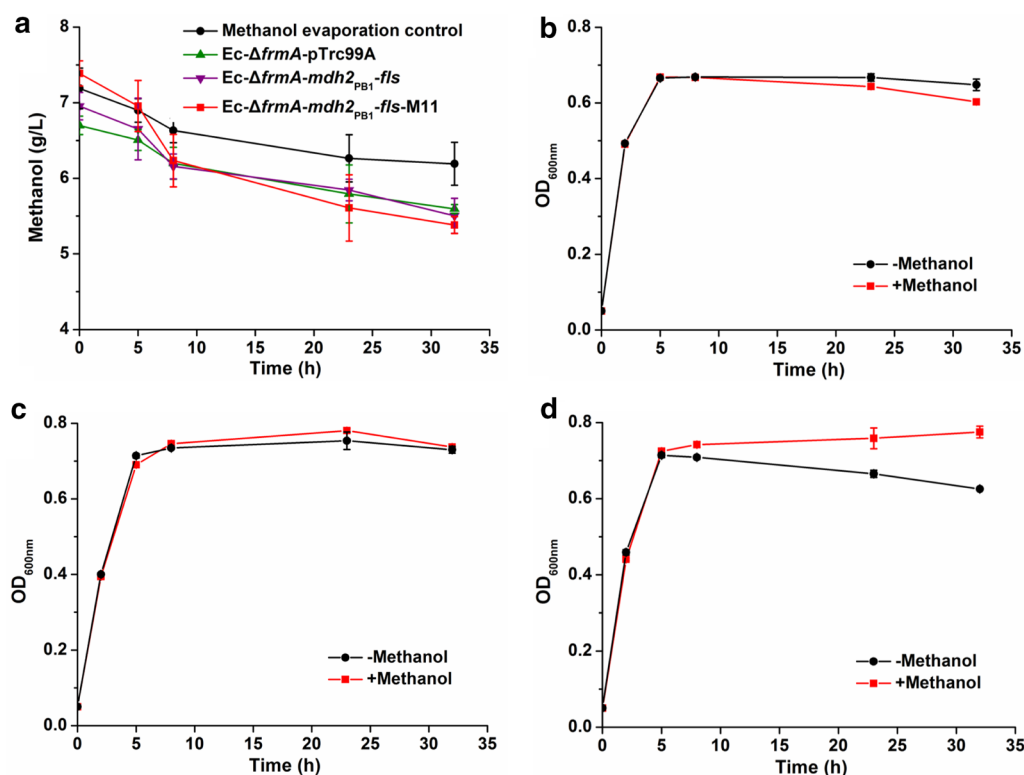

Fig. 2 Methanol consumption and growth characteristics of *E. coli* strains. **a** Methanol evaporation and consumption. **b** Cell growth of strain Ec-Δ*frmA*-pTrc99A. **c** Cell growth of strain Ec-Δ*frmA-mdh2*_{PB1}-*fls*. **d** Cell growth of strain Ec-Δ*frmA-mdh2*_{PB1}-*fls*-M11. Cells were cultured in M9 minimal medium supplemented with 1 g/L yeast extract or M9 minimal medium supplemented with 1 g/L yeast extract and methanol. Error bars indicate standard deviation ($n = 3$)

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^{13}C-methanol-labeling experiment was performed. When ^{13}C-methanol was used as a carbon source, ^{13}C-labeled amino acids in biomass including alanine, aspartic acid, glutamic acid, phenylalanine, proline, glycine, lysine, serine, threonine, tyrosine, and ^{13}C-labeled citric acid were detected (Fig. 3a; Additional file 1: Table S4). It has been reported that amino acids measurement could provide isotopic labeling information about eight crucial precursor metabolites in the central metabolism (You et al. 2012). The presented results showed that biosynthesis of key intermediates of glycolysis, PP pathway and TCA cycle, including 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA, α-ketoglutarate, oxaloacetate, and erythrose 4-phosphate, withdrew carbon from ^{13}C-methanol.

Adaptive evolution of the engineered *E. coli* to improve methanol utilization

To further improve the microbial performance in methanol medium and screen methanol-utilizing mutants, adaptive evolution based on GREACE (genome replication engineering assisted continuous evolution) was conducted (Luan et al. 2013). A proofreading-defective element of the DNA polymerase of *E. coli* (ε subunit encoded by *dnaQ* gene) was expressed in strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls* to introduce random mutations into the genomic DNA during continuous passage cultivation in LB medium. For each passage, cells were transferred into M9 minimal medium supplemented with methanol to enrich potential mutants with improved cell growth on methanol. Mutants were then isolated from the culture and a mutant with the best cell growth on methanol was isolated and designated as Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11 (Additional file 1: Figure S1). When mutant Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11 was cultivated in

M9 minimal medium supplemented with 1 g/L yeast extract and methanol, 2 g/L methanol was consumed, which was more than that consumed by its parent strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*. It was noticed that biomass of the mutant declined after 5 h and addition of methanol helps maintain the biomass (Fig. 2d). We speculated that such decline in cell growth was caused by the random mutations introduced by GREACE. ^{13}C-methanol-labeling experiment was then conducted and the results validated that mutant Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11 assimilated more ^{13}C-methanol into biomass (Fig. 3b; Additional file 1: Table S5). The results demonstrated that coupling of metabolic engineering and adaptive evolution was an enabling strategy to endow microorganisms with the ability to utilize methanol.

Synthetic methylotrophs have been constructed by heterogenous expressing MDH and RuMP genes (Pfeifenschneider et al. 2017). RuMP cycle depends on regenerating the formaldehyde acceptor Ru5P, which requires high coordination of many enzymes involved in formaldehyde assimilation and PP pathway (Whitaker et al. 2015). On the contrary, the linear formaldehyde assimilation pathway used in this study only requires one enzyme FLS and directly produces C3 intermediate DHA, which could be a great advantage for pathway engineering. Previous and the present studies revealed that constructing synthetic methylotrophs was far more complicated than complementing metabolic pathways where several crucial factors need to be considered, such as how to keep the intracellular formaldehyde concentration below the toxicity threshold (Witthoff et al. 2015) and how to balance the reducing equivalent generated by methanol oxidation (Price et al. 2016). In this case, combining metabolic engineering and adaptive evolution could be an easy strategy to prepare a desirable mutant that assimilates methanol efficiently.
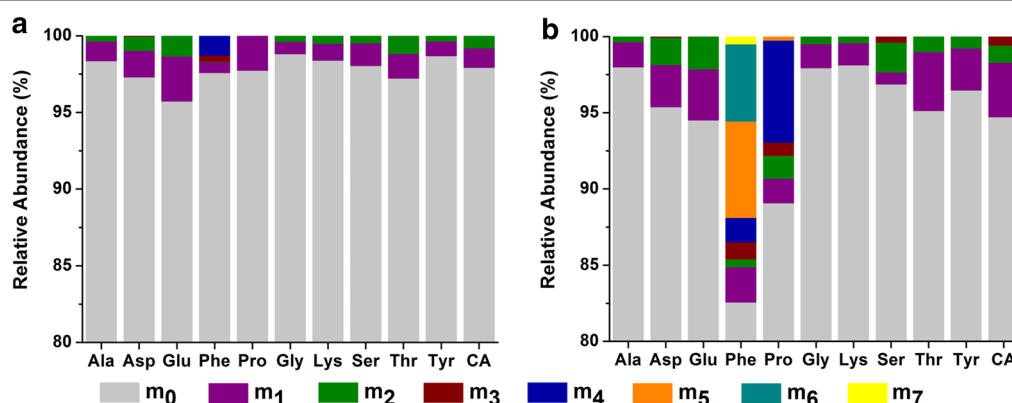

Fig. 3 Biomass mass isotopomers of strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls* (**a**) and strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11 (**b**) using ^{13}C-labeled methanol. *CA* citric acid. Values are corrected for natural abundance. Data are the means from three parallel experiments

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By using such a combined strategy, improved methanol assimilation was obtained in the mutant Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11. Whole-genome resequencing revealed that no mutation was introduced into the plasmid, which was consistent with the unchanged MDH activity (Fig. 1b). Meanwhile, 66 missense, synonymous, and intergenic mutations that covered amino acid transport and metabolism, signal transduction, cell wall/membrane/envelope biogenesis, etc. were discovered (Additional file 2: Table S6). Further investigation of these mutations will likely elucidate key factors of methanol utilization in synthetic methylotrophs.

Conclusions

In this study, an artificial linear methanol assimilation pathway was functionally assembled in *E. coli*. Methanol utilization by the engineered strain was facilitated and further improved by adaptive evolution. ^{13}C-methanol-labeling experiment revealed the methanol incorporation into cellular biomass. This study is the first demonstration of applying the linear methanol assimilation pathway for biological conversion of methanol. The combined strategy of metabolic engineering and adaptive evolution is also a useful approach to endow platform strains with the ability to utilize other C1 resources such as the main component of natural gas, methane.

Additional files

Additional file 1: Additional methods. Figure S1. Adaptive evolution by genome replication engineering assisted continuous evolution (GREACE) and screening of methanol-utilizing mutants. **Table S1.** Bacterial strains and plasmids used in this study. **Table S2.** Sequences of primers used in this study. **Table S3.** Sequences of *mdh3*$_{MGA3}$, *mdh2*$_{PB1}$, and *fls* genes. **Table S4.** Biomass mass isotopomers of strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls* using ^{13}C-methanol as substrate. Values are corrected for natural abundance. **Table S5.** Biomass mass isotopomers of strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11 using ^{13}C-methanol as substrate. Values are corrected for natural abundance.

Additional file 2: Table S6. List of mutations identified using whole genome resequencing of strain Ec-Δ*frmA-mdh2*$_{PB1}$-*fls*-M11.

Abbreviations

RuMP: ribulose monophosphate; Ru5P: ribulose-5-phosphate; PP pathway: pentose phosphate pathway; MDH: methanol dehydrogenase; FLS: formolase; DHA: dihydroxyacetone.

Authors' contributions

YW, JL, and JS conceived and designed the experiments. XW, YW, QL, and ZZ performed the experiments. XW, YW, and JL analyzed the data. PZ, FL, and JS contributed reagents and analytic tools. YW, PZ, FL, and JS wrote the paper. All authors read and approved the final manuscript.

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