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REVIEW PAPER

Reconstruction of Methanol and Formate Metabolic Pathway in Non-native Host for Biosynthesis of Chemicals and Biofuels

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Abstract One-carbon feedstock such as methanol and formate has attracted much attention as carbon substrate of industrial biotechnology for production of value-added chemicals and biofuels. Productivity improvement of natural one-carbon metabolic pathways in native hosts such as methanotrophs is somewhat difficult due to inefficient genetic tools and low specific growth rate. As an alternative, metabolic engineering can create new and efficient metabolic pathways of one-carbon substrate that can be readily transferred to non-native hosts. In this paper, recent progresses in protein and metabolic engineering for creation of methanol and formate-utilizing synthetic pathways based on RuMP cycle and formolase are reviewed. Perspectives on one-carbon metabolic pathway engineering in non-native host are also discussed.

Keywords: methanol, formate, formolase, methanol dehydrogenase, RuMP cycle

1. Introduction

One-carbon compounds such as methane, methanol and formate are promising substrates for industrial biotechnology. Especially, methane is considered as a next-generation carbon feedstock due to its abundant availability and low price [1]. Methanotrophs can utilize methane as sole carbon and energy source, and thus they can be used as the biocatalyst for methane bioconversion [2]. In the first step, methane is oxidized to methanol by two kinds of methane monooxygenase (MMO) including particulate MMO (pMMO) and soluble MMO (sMMO) [3]. Interestingly, there has been no report on successful heterologous expression of MMO in non-native hosts to date, which preclude the possibility of using preexisting industrial hosts such as *Escherichia coli* for bioconversion of methane to value-added chemicals and biofuels. Hydroxylase unit of sMMO has not been heterologously expressed as functionally active form [4-6]. As a result, full activity of sMMO has not been observed in non-native hosts up to now. Similarly, there was little or no pMMO activity when expressed in different hosts including *E. coli*, *Enterobacter aerogenes*, *Rhodococcus erythropolis*, and *Pseudomonas putida*, most probably due to incorrect folding of pMMO [7,8]. In addition, the expression of pMMO in *E. coli* seemed to be toxic to host cells [9,10].

Besides methane monooxygenase, P450-oxygenase has been engineered using protein engineering and directed evolution to accept methane as the substrate. However, the engineered P450 monooxygenase did not convert methane into methanol [11]. An engineered cytochrome P450 BM3 variant has catalyzed the conversion of ethane to ethanol, but not methane to methanol [12]. Thus, heterologous reconstruction of methane metabolic pathway in non-native hosts has not been realized to date due to unavailability of methane-converting heterologous enzyme in non-native hosts.

Methanol is an attractive carbon substrate because of its high production volume and low market price. Bioconversion of methanol to value-added chemicals has been mainly conducted using wild-type methylotrophic strains [13]. As an example, poly-β-hydroxybutyrate (PHB) up to 130 g/L was obtained using wild-type methylotrophs [14], and PHB was accumulated up to 60% of total biomass [15]. Recently, reconstruction of methanol metabolic pathway

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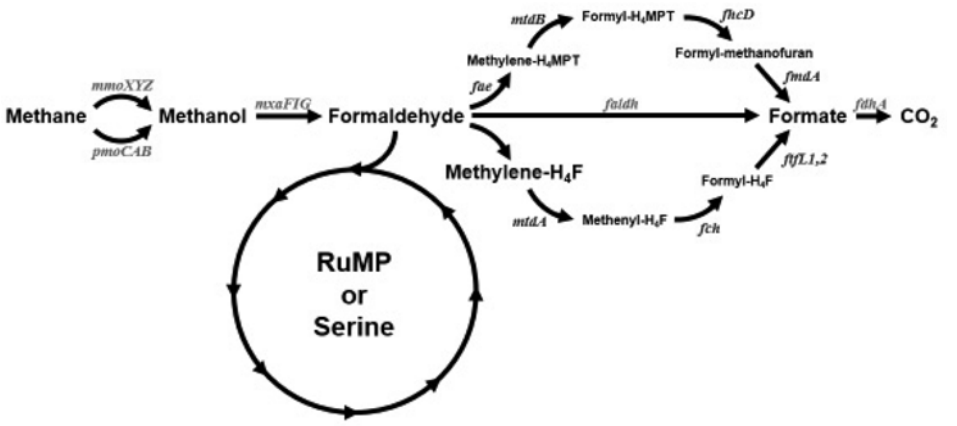

Fig. 1. Simplified metabolic pathway of methane to carbon dioxide. mmo: Soluble methane monooxygenase; pmo: Particulate methane monooxygenase; mxa: Methanol dehydrogenase; fae: Tetrahydromethanopterin hydrolyase; mtdB: Methenyl-H_4MPT cyclohydrolase; fhc: Formyl-methanofuran-H_4MPT formyltransferase; fmd: Formylmethanofurane dehydrogenase; mtdA: Methylene-H_4F dehydrogenase; fch: Methenyl-H_4F cyclohydrolase; ftf: Formyl-H_4F cycloligase; faldh: Formaldehyde dehydrogenase; fdh: Formate dehydrogenase.

has been investigated in non-native hosts such as *P. putida* [16], *E. coli* [17], *Corynebacterium glutamicum* [18,19], which provides new opportunities for producing chemicals from methanol using preexisting industrial hosts.

Formate is also produced at low price from industrial waste gas, carbon monoxide, with sodium hydroxide. Formate can be assimilated as carbon substrate through central metabolism of wild-type methylotrophs by a small quantity. Recently, a synthetic assimilation pathway of formate as a glycolysis intermediate using computationally designed formolase (FLS) has been successfully constructed in *E. coli* [20]. Introduction of novel chemomimetic enzyme, formolase, created the reconstruction of formate metabolic pathway in non-native hosts [21]. In this paper, we reviewed on recent progresses on the reconstruction of metabolic pathway for the conversion of methanol and formate to chemicals in non-native hosts. Perspectives on methanol- and formate-metabolic pathway engineering in non-native hosts are also discussed.

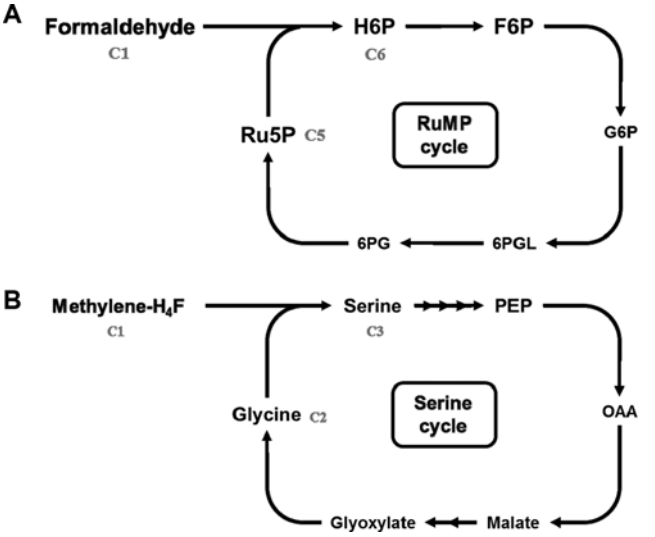

Fig. 2. Simplified (A) RuMP and (B) serine cycle. H6P: Hexulose-6-phosphate; F6P: Fructose-6-phosphate; G6P: Glucose-6-phosphate; 6PGL: 6-Phosphogluconolactone; 6PG: 6-Phosphogluconase; Ru5P: Ribulose-5-phosphate; PEP: Phosphoenolpyruvate; OAA: Oxaloacetate.

2. Overview of C1 Metabolic Pathway

Methanotrophic bacteria assimilate one-carbon substrate at the oxidation level of formaldehyde. Methanotrophic bacteria use MMOs to convert methane to methanol at the first step and then methanol is oxidized to formaldehyde by methanol dehydrogenase (MDH) [3]. Whereas gram-negative methylotrophs as well as methanotrophs use pyrroloquinoline quinone (PQQ)-dependent MDH along with PQQ biosynthetic pathway to oxidize methanol [3], gram-positive bacteria such as *Bacillus* strains use NAD-dependent cytoplasmic MDH [22]. Formaldehyde is oxidized to formate *via* tetrahydrofolate (H_4F) or tetrahydromethanopterin (H_4MPT)-mediated pathways and then formate is finally oxidized to carbon dioxide by formate dehydrogenase (Fig. 1).

Methanotrophs and methylotrophs assimilate formaldehyde into central metabolic pathway by using ribulose monophosphate (RuMP) cycle or serine cycle (Fig. 2). Hexulose 6-phosphate synthase (HPS) and hexulose 6-phosphate isomerase (PHI) are two key enzymes in formaldehyde fixation phase of RuMP cycle. In the first step of RuMP pathway, condensation of formaldehyde with ribulose 5-phosphate (Ru5P) generates hexulose 6-phosphate (H6P), and then isomerized to fructose 6-phosphate (F6P) [23].

Ribulose 5-phosphate is generated through several possible series of reactions to complete RuMP cycle. F6P is assimilated through either Entner-Doudoroff (EDD) pathway or Embden-Meyerhof-Parnas (EMP) pathway. In methanotrophs, EDD pathway is expected to occur mainly based on the relative activities of relevant enzymes [23]. F6P is converted to 6-phosphate gluconate, and then to 2-keto-3-deoxy-6-phosphogluconate (KDPG) by 6-phosphogluconic dehydratase in EDD pathway. KDPG is cleaved to glyceraldehyde 3-phosphate and pyruvate. In EMP pathway, F6P is converted to fructose 1,6-bisphosphate (FBP) by phosphofructokinase, FBP is subsequently cleaved to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) by aldolase, and finally to pyruvate [24,25]. Recently, it was reported that the dominant pathway for generating pyruvate was the EMP pathway up to 75% of pyruvate in *Methylomicrobium buryatense* strain 5G by ^{13}C-labeling analysis [25].

Serine cycle is initiated by synthesizing one molecule serine from glycine (C2) and methylene-H_4F (C1) to form serine (C3) by serine hydroxymethyltransferase [26]. Serine is converted to hydroxypyruvate by serine-glyoxylate aminotransferase. The intermediate, 2-phosphoglycerate is assimilated to cell material during serine cycle. One mole of carbon dioxide is fixed to phosphoenolpyruvate to generate oxaloacetate (C4) by phosphoenolpyruvate carboxylase. Malyl-CoA is cleaved into glyoxylate and acetyl-CoA by malyl-CoA lyase. In the end of serine cycle, NH_2-group is regenerated during the regeneration of glycine from glyoxylate catalyzed by serine-glyoxylate aminotransferase.

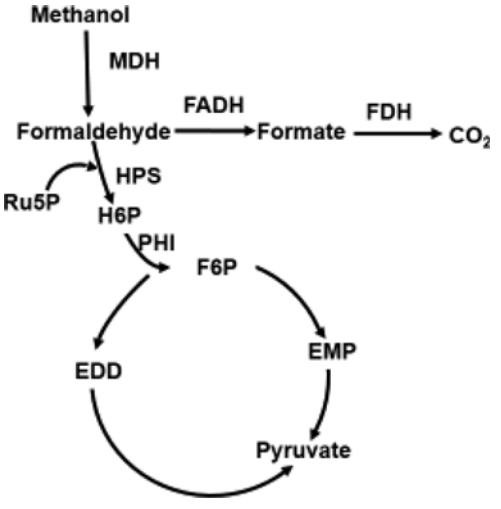

Fig. 3. Reconstruction of methanol assimilation pathway in non-native host. MDH: Methanol dehydrogenase; HPS: Hexulose 6-phosphate synthase; PHI: Hexulose 6-phosphate isomerase; FADH: Formaldehyde dehydrogenase; FDH: Formate dehydrogenase; Ru5P: Ribulose-5-phosphate; H6P: Hexulose 6-phosphate; F6P: Fructose 6-phosphate; EMP: Embden-Meyerhof-Parnas; EDD: Entner-Doudoroff.

Oxaloacetate and malate are interconnected with tricarboxylic acid cycle (TCA cycle). Serine cycle is also interconnected with ethylmalonyl-CoA cycle (EMC cycle). Two moles acetyl-CoA form serine cycle and one mole carbon dioxide are converted to ethylmalonyl-CoA (C5), and finally for regeneration of glyoxylate to complete serine cycle. TCA cycle and EMC cycle are also interconnected. Methylmalonyl-CoA (C4) of EMC cycle is connected to succinyl-CoA of TCA cycle. Besides, the other methylated compounds such as monohalogenated methanes, dihalogenated methanes, methylated sulfur species also serve as substrates for methylotrophic bacteria, and their metabolisms are enabled by well-characterized specific modules which have been reported previously [27].

3. Reconstruction of RuMP-based Methanol Metabolic Pathway in Non-native Hosts

Engineering of RuMP-based methanol metabolic pathway in non-native hosts has been first conducted in *P. putida* [16]. The HPS and PHI genes of *B. brevis* were introduced into *P. putida* for assimilation of toxic formaldehyde intermediate generated from methanol. Even though methanol dehydrogenases was not identified in *P. putida* genome sequence, low methanol dehydrogenase activity was detected from a side activity of alcohol dehydrogenase with broad substrate specificities. This study gave a hint on how to improve methanol utilization by increasing the endogenous alcohol dehydrogenase activity or by expressing a heterologous methanol dehydrogenase. Thus, engineering strategy for implementing methanol metabolic pathway into non-native hosts was based on expressing a set of "methylotrophy genes" including methanol dehydrogenases for utilizing methanol and HPS-PHI for establishing complete RuMP cycle (Fig. 3).

Recently, RuMP-based reconstruction of methanol metabolic pathway in non-native hosts such as *E. coli* [17] and *C. glutamicum* has been demonstrated [18,19]. As mentioned above, methylotrophic bacteria are able to utilize methanol at the first step of metabolism using methanol dehydrogenase. NAD-dependent methanol dehydrogenase from gram-positive methylotrophic bacteria *B. methanolicus* was considered as a good candidate for methanol oxidation in non-native hosts [28]. NAD-dependent MDHs are active under both aerobic and anaerobic conditions, whereas the activity of PQQ-dependent MDHs would be limited to aerobic condition [28]. Recently, methanol dehydrogenases from *B. methanolicus* have been used in *E. coli* and *C. glutamicum* for methanol utilization [17-19]. *B. methanolicus* MGA3 is known to express MDH, MDH2, and MDH3, while *B. methanolicus* PB1 expresses MDH, MDH1, and

MDH2 [29]. Among those genes encoding methanol dehydrogenase from *B. methanolicus* strains MGA3 and PB1, MDH from *B. methanolicus* MGA3 was shown to possess the highest activity in recombinant *C. glutamicum* [19] and MDH2 from *B. methanolicus* MGA3 was most suitable for expression in *E. coli* [17].

The RuMP pathway is divided into three main parts including fixation, cleavage and rearrangement [23]. HPS and PHI are two key enzymes in fixation phase and play an important role in formaldehyde assimilation pathway. Overexpression of those enzymes in both of homologous and heterologous hosts leaded to enhance formaldehyde incorporation. Homologous expression of HPS and PHI genes in *B. methanolicus* improved formaldehyde tolerance and growth rate on methanol, compared to the wild-type strain [30]. Among several HPS-PHI gene clusters from different microbial sources, HPS-PHI genes from *B. methanolicus* MGA3 showed best effectiveness in *E. coli* [17]. For expression of HPS-PHI in *C. glutamicum*, HxlA and HxlB from *B. subtilis* showed higher activities *in vitro* as well as *in vivo* [19]. Furthermore, in order to prevent formaldehyde detoxification *via* the linear formaldehyde dissimilation pathways, endogenous aldehyde dehydrogenase (ald) and mycothiol-dependent formaldehyde dehydrogenase (fadH) in *C. glutamicum* [18] and endogenous glutathione-dependent formaldehyde dehydrogenase in *E. coli* were deleted [17]. Labeling experiments using ^{13}C-methanol indicated that the engineered *E. coli* showed up to 40% incorporation of methanol into central metabolites and the presence of the endogenous glutathione-dependent formaldehyde oxidation pathway of *E. coli* did not adversely affect the methanol conversion rate [17]. In comparison, the oxidation of formaldehyde to CO_2 was strongly affected in *C. glutamicum* Δald ΔfadH mutant and the mutation resulted in improvement of formaldehyde assimilation *via* RuMP cycle [18,19]. The engineered *C. glutamicum* expressing MDH and HPS-PHI genes grew well in the presence of methanol as well as formaldehyde, and converted methanol to cadaverine with an average methanol consumption rate of 1.7 ± 0.3 mM/h. However, until now, no case of growth with only methanol was observed because of limitation in the regeneration of HCHO-accepting five-carbon sugar phosphate [28]. This can be overcome *via* heterologous expression of C5 regeneration-related genes or replacing the native promoters of pentose phosphate pathway (PPP) with inducible promoters. Besides, deletion of phosphoglucose isomerase which is important for cyclic formaldehyde dissimilation but not required for RuMP cycle or overexpression of glucose-6-phosphate dehydrogenase and fructose 1,6-bisphosphatase which leads to overexpression of PPP enzymes might enable cells to grow on methanol [28]. With respect to patent, Eleftherios et al. have claimed the expression of MDH and HPS-PHI genes for production of liquid fuels and chemicals from methanol, although no quantitative data have been reported for those claims [28]. All these results clearly represent that the reconstruction of RuMP-based methanol metabolic pathway is promising approach for methanol-based production of chemical and fuels in non-native hosts like preexisting industrial hosts like *E. coli*.

4. Reconstruction of Formolase-based Formate Metabolic Pathway in Non-native Hosts

Compared to the reconstruction methanol assimilation pathway, relevant works for a reconstruction of formate assimilation pathway in non-native host have been recently conducted. The most important achievement was the development of new functional enzyme, formolase (FLS), using computational design [20]. FLS catalyzes the formose reaction, that is, carboligation of three formaldehyde molecules into one three-carbon dihydroxyacetone (DHA). Thus, FLS converts one-carbon formate substrate into a

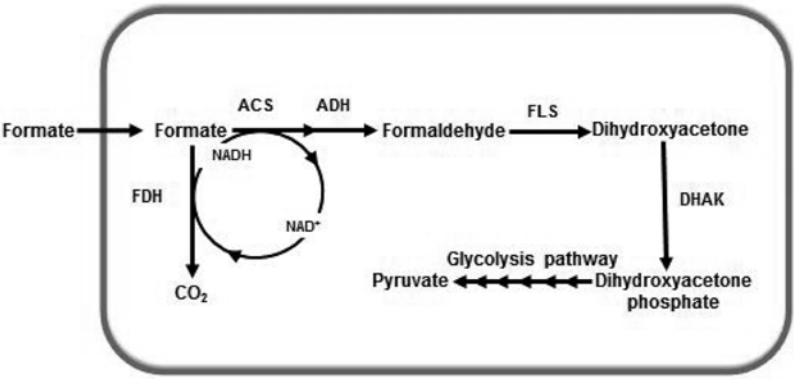

Fig. 4. Reconstruction of formate assimilation pathway in non-native host. FDH: Formate dehydrogenase; ACS: Acetyl-CoA synthase, ADH: Acetaldehyde dehydrogenase; FLS: Formolase; DHAK: Dihydroxyacetone kinase.

three-carbon glycolysis intermediate, and FLS-mediated formolase pathway enables a new thermodynamically favorable carbon fixation pathway with only five reaction steps. Benzaldehyde lyase (BAL) was identified as a platform protein, and its binding pocket was redesigned to increase specificity and activity for formose reaction using computational protein design. The resulting FLS had a catalytic efficiency of 4.7/M/sec. Mass spectrometry revealed that dihydroxyacetone and glycolaldehyde were formed by FLS and the partitioning between two products was dependent on the formaldehyde concentration [20].

The formolase-based synthetic metabolic pathway assimilates formate into biomass through DHA in only five steps (Fig. 4) [21]. After carbon dioxide is converted to formate, *e.g.*, through electrochemical means, formate is partitioned to provide both carbon flux to formaldehyde and NADH. Pathway for formate to formaldehyde is catalyzed by acylating acetaldehyde dehydrogenase and acetyl-CoA synthase. For the conversion of three one-carbon formaldehyde into one DHA, the formolase catalyzes this carboligation reaction. DHA is phosphorylated to DHAP (dihydroxyacetone phosphate) and this intermediate is plugged into many preexisting production pathways for chemicals and biofuels [31-36]. The formose pathway can be one of the most efficient pathways ever realized.

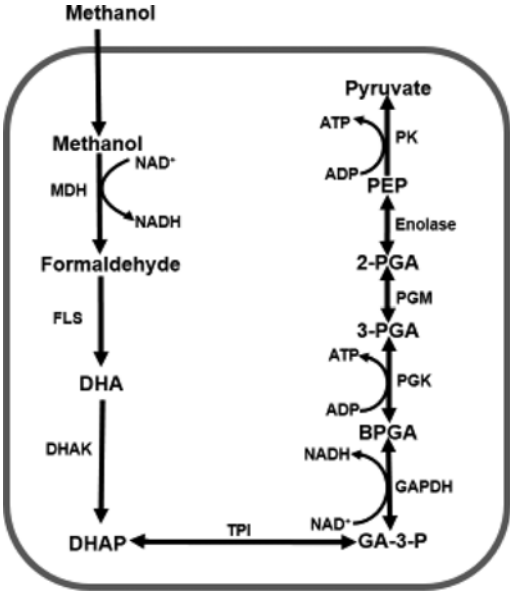

Fig. 5. Simplified scheme of the reconstruction of formolase-based methanol assimilation pathway in non-native host. MDH: Methanol dehydrogenase; FLS: Formolase; DHA: Dihydroxyacetone; DHAK: Dihydroxyacetone kinase; DHAP: Dihydroxyacetone phosphate; TPI: Triosephosphate isomerase; GA-3-P: D-glyceraldehyde 3-phosphate; GAPGH: Glyceraldehyde 3-phosphate dehydrogenase; BPGA: 1,3-Bisphosphoglycerate; PGK: Phosphoglycerate kinase; 3-PGA: 3-Phosphoglycerate; PGM: Phosphoglycerate mutase; 2-PGA: 2-Phosphoglycerate; PEP: Phosphoenolpyruvate; PK: Pyruvate kinase.

5. Perspectives

Methanol can be utilized by non-native hosts through RuMP pathway by expression of three genes of MDH, HPS and PHI. However, because of limitation in the regeneration of 5-carbon sugar phosphate when grown on C1 substrates, the engineered hosts could not sustainably use methanol as the sole carbon source [17-19,28]. Heterologous expression of the C5-regeneration genes or overexpression of PPP enzymes might enable non-native hosts to grow on methanol only. With respect to formate utilizing pathway in non-native host, computational design-assisted protein engineering for the creation of a novel enzyme, FLS, have facilitated the creation of new functional enzyme for a new biosynthetic pathway, the formolase pathway [20,21]. Unlike other native one-carbon assimilation pathways, the formolase pathway is predicted to be the shortest, linear, not oxygen sensitive and more efficient carbon assimilation with less backward flux. However, FLS has a very low catalytic efficiency, even though its activity was 100-fold increased for formose reaction and its specificity was 10 million-fold enhanced based on the relative specificity between BAL and FLS [21]. Thus, the catalytic efficiency of FLS needs to be further enhanced using protein engineering technique.

Formolase-based methanol assimilation into glycolytic pathway will be useful for the production of target products whose synthetic pathway requires many reducing power like NADH because the oxidation of methanol by MDH generates NADH (Fig. 5). Simple recruit of two genes of MDH and FLS can complete an efficient and linear biosynthetic pathway from methanol to glycolytic intermediate. This synthetic pathway can be liked up with many preexisting ready-production pathway, mainly of metabolically engineered *E. coli*–based cell factory for the production of chemicals and biofuels. Protein engineering of methane monooxygenase that make MMO can work properly in non-native hosts like *E. coli* will expand this synthetic metabolic pathway to methane substrate, and the production of valuable chemicals and biofuels from methane using non-native host will be possible.

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