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Enhanced performance of the methylerythritol phosphate pathway by manipulation of redox reactions relevant to IspC, IspG, and IspH

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ABSTRACT

The 2C-methyl-D-erythritol 4-phosphate (MEP) pathway is a carbon-efficient route for synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the building blocks of isoprenoids. However, practical application of a native or recombinant MEP pathway for the mass production of isoprenoids in *Escherichia coli* has been unsatisfactory. In this study, the entire recombinant MEP pathway was established with plasmids and used for the production of an isoprenoid, protoilludene. *E. coli* harboring the recombinant MEP pathway plasmid (ME) and a protoilludene synthesis pathway plasmid (AO) produced 10.4 mg/L of protoilludene after 48 h of culture. To determine the rate-limiting gene on plasmid ME, each constituent gene of the MEP pathway was additionally overexpressed on the plasmid AO. The additional overexpression of IPP isomerase (IDI) enhanced protoilludene production to 67.4 mg/L. Overexpression of the Fpr and FldA protein complex, which could mediate electron transfer from NADPH to Fe–S cluster proteins such as IspG and IspH of the MEP pathway, increased protoilludene production to 318.8 mg/L. Given that it is required for IspC as well as IspG/H, the MEP pathway has high demand for NADPH. To increase the supply of NADPH, a NADH kinase from *Saccharomyces cerevisiae* (tPos5p) that converts NADH to NADPH was introduced along with the deletion of a promiscuous NADPH-dependent aldehyde reductase (YjgB) that consumes NADPH. This resulted in a protoilludene production of 512.7 mg/L. The results indicate that IDI, Fpr-FldA redox proteins, and NADPH regenerators are key engineering points for boosting the metabolic flux toward a recombinant MEP pathway.

1. INTRODUCTION

Isoprenoids are a large family of over 55,000 valuable natural compounds with potential as pharmaceuticals, insecticides, fragrances, and ideal fuel alternatives (McGarvey and Croteau, 1995; Peralta-Yahya and Keasling, 2010). They are derived from the universal building blocks, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). These building blocks are synthesized from two well-known biosynthetic pathways, namely the mevalonate (MVA) pathway and the 2C-methyl-D-erythritol 4-phosphate (MEP) (also known as DXP) pathway (Goldstein and Brown, 1990; Rohmer, 1999). As compared to the MVA pathway, the MEP pathway is theoretically more efficient for isoprenoid production, especially in terms of carbon efficiency (Partow et al., 2012). The MEP pathway, which is composed of seven enzymes (Dxs, IspC, IspD, IspE, IspF, IspG, and IspH), synthesizes IPP and DMAPP from pyruvate and D-glyceraldehyde-3-phosphate in a ratio of 5–6:1 (Rohdich et al., 2003) (Fig. 1A). This ratio can be shifted to 3:7 by overexpression of an isopentenyl diphosphate isomerase (IDI) catalyzing the reversible isomerization of IPP to DMAPP (Rohdich et al., 2003).

In recent decades, microbial engineering has emerged as a promising method for sustainable mass production of rare natural compounds including isoprenoids. Synthesis of IPP and DMAPP for isoprenoids production has been augmented in some model microorganisms such as *Escherichia coli* and *Bacillus subtilis* by manipulation of their endogenous MEP pathways (Kim and Keasling, 2001; Xue and Ahring, 2011). Early studies suggest that Dxs, IspC, and IDI are the key enzymes of the MEP pathway to enhance production of isoprenoids from *E. coli* (Kajiwara et al., 1997; Kim and Keasling, 2001; Reiling et al., 2004). However, further increase in isoprenoid production has not been achieved by simple overexpression of several key enzymes of the MEP

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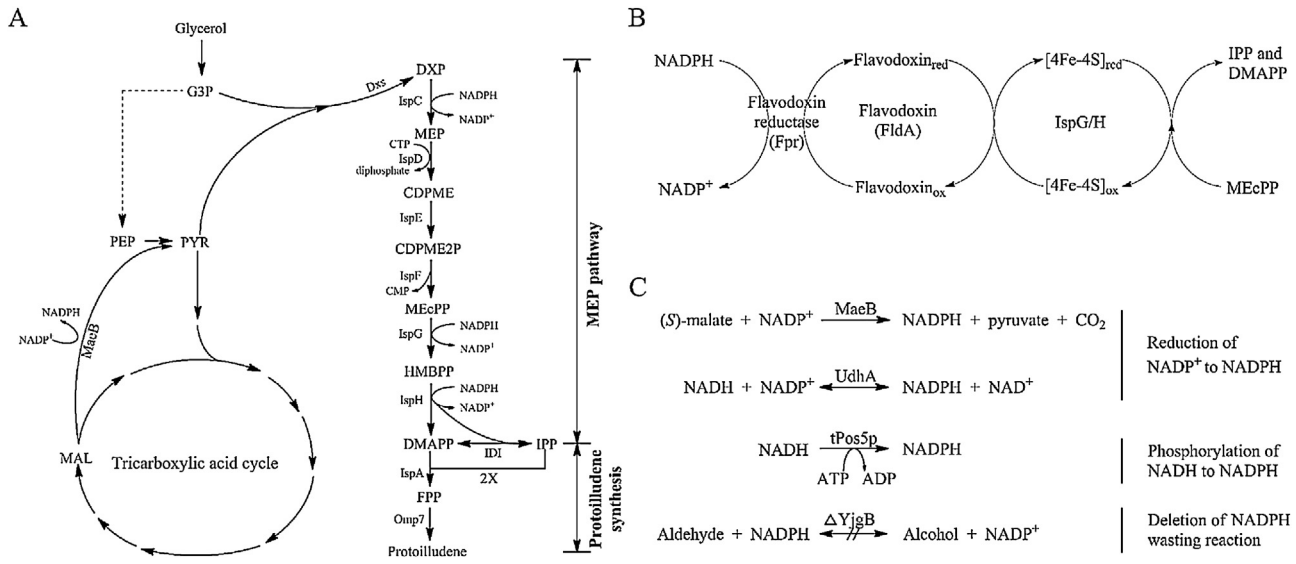

Fig. 1. MEP pathway and its relevant metabolism for protoilludene synthesis. (A) Synthesis of protoilludene from glycerol through the MEP pathway. PYR and G3P, which can be synthesized from glycerol, are converted into the isoprenoid building blocks of IPP/DMAPP through the MEP pathway. IPP/DMAPP is used for protoilludene synthesis by IspA and Omp7. (B) Reduction of Fe-S clusters of IspG/H by Fpr-FldA complex and NADPH. (C) Enhancing availability of NADPH for MEP pathway by reduction of $NADP^+$ to NADPH, phosphorylation of NADH to NADPH, and deletion of an unnecessary reaction of NADPH. Abbreviations used here are as follows. Metabolites are G3P, D-glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; MAL, malate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDPME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDPME2P, 2-phospho-4-diphosphocytidyl-2-C-methyl-D-erythritol; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-butenyl- 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; and FPP, farnesyl diphosphate. Enzymes are Dxs, DXP synthase; IspC, DXP reductoisomerase; IspD, CDPME synthase; IspE, CDPME kinase; IspF, MEcPP synthase; IspG, HMBPP synthase; IspH, HMBPP reductase; IDI, IPP isomerase; IspA, FPP synthase; Omp7, *Omphalotus olearius* protoilludene synthase; MaeB, $NADP^+$-dependent malate dehydrogenase; UdhA, pyridine nucleotide transhydrogenase; tPos5p, NADH kinase of *S. cerevisiae* lacking of mitochondrial targeting sequence; and YjgB, NADPH-dependent aldehyde reductase.

pathway because the metabolic engineering-confined MEP pathway itself is blocked by an unknown metabolic or regulatory obstacle (Martin et al., 2003). Metabolite profiling of *E. coli* overexpressing Dxs, IDI, IspD, and IspF showed a large accumulation of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP), the substrate of IspG (Zhou et al., 2012). The surplus of MEcPP was not reduced by simple overexpression of its downstream enzymes, IspG and IspH (Zou et al., 2013). Thus, the catalytic activities of IspG and H are suspected to be limited to either nonfunctional expression or an insufficient redox recycling reaction via flavodoxin (Fig. 1B).

IspG and H are iron–sulfur (Fe–S) cluster proteins containing a (4Fe–4S) prosthetic group (Wolff et al., 2003). Multiple Fe–S cluster building and trafficking pathways are involved in the maturation processes of IspG and H (formation of holo-IspG/H) (Py and Barras, 2010). ISC (iron–sulfur cluster) and SUF (sulfur utilization factor) help Fe–S proteins to acquire Fe–S clusters in *E. coli* (Py and Barras, 2010). Co-overexpression of ISC and IspG/H increased the functional formation of IspG/H, suggesting enhanced Fe–S cluster acquisition (Grawert et al., 2004; Zepeck et al., 2005). However, there was either a severe reduction or only a negligible increase in amorphadiene production after the overexpression of ISC proteins in strains with overexpression of a recombinant MEP pathway (Zou et al., 2013). The additional Fe–S cluster acquisition of IspG/H is not suspected to be a critical engineering point for the MEP pathway.

Both IspG and IspH reactions have to be coupled with a reduction system, which shuttles electrons from a reduced donor to the oxidized Fe–S cluster of IspG/H (Rohdich et al., 2003). IspG and IspH are known to receive electrons from the reduced form of flavodoxin I encoded by the *fldA* gene (Puan et al., 2005). Flavodoxin I is reduced by a flavodoxin/ferredoxin $NADP^+$ reductase, encoded by the *fpr* gene, using NADPH as the preferred electron donor rather than NADH (Grawert et al., 2004; Wolff et al., 2003). FldA, Fpr, and NADPH thus compose an efficient *E. coli* electron transfer system, known as the NADPH-Fpr-FldA reducing system, responsible for the activation of IspG/H (Grawert et al., 2004; Zepeck et al., 2005) (Fig. 1B).

NADPH is also indispensable to the maturation process of all Fe–S cluster proteins during Fe–S cluster trafficking (Kim et al., 2013; Yan et al., 2013). An oxidized ferredoxin (Fdx) receives one electron from NADPH and delivers the electron to an Fe–S cluster via physical interactions with both Fpr and the ISC machinery protein (IscS, cysteine desulfurase) (Kim et al., 2013; Yan et al., 2013). The electron transfer pathway composed of NADPH, Fpr, and Fdx is thereby required for the maturation of IspG/H. Taken together, NADPH plays a critical role in both the maturation and activation processes of IspG/H. Besides IspG and IspH, another MEP pathway enzyme, IspC, also requires NADPH as the co-factor for its catalytic activity (Rohdich et al., 2003; Takahashi et al., 1998). The tight NADPH-dependent activity of the MEP pathway highlights the potential importance of engineering NADPH regenerators.

In this study, we focused on engineering the activation process of IspG/H and NADPH regenerators to establish a robust recombinant MEP pathway in *E. coli*.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

E. coli DH5α (F^-,Φ80d *lacZ*ΔM15, Δ(*lacZYA-argF*) U169, *deoR*, *recA*1, *endA*1, *hsdR*17($r_K^- m_K^+$), *phoA*, *supE*44, λ^-, *thi*-1; ATCC 98040) was used as the parent strain. For knockout of *yjgB* from the chromosome of *E. coli* DH5α, a FRT-flanked kanamycin resistance cassette was amplified from plasmid pKD13 (Baba et al., 2006) with a primer pair of yjgB-KO-F/yjgB-KO-R and transformed into DH5α cells harboring plasmid pRedET for homologous recombination according to the instructions of the Quick and Easy Red/ET *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg; Cat. No. K006). The kanamycin resistance marker was removed from the chromosome to finally establish *E. coli* DH5αΔ*yjgB*. Antibiotics (100 mg/L