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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 towards a recombinant MEP pathway.

***Keywords*:** MEP pathway; isoprenoid; *Escherichia coli*; Fe-S cluster protein;

NADPH

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 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 cofactor 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 ampicillin, 50 mg/L kanamycin, and 25 mg/L gentamycin) were added as required in experiments. Both seed and main cultures were aerobically performed in glass tubes (2.5 cm × 15 cm) with plastic caps. For seed culture, individual colonies grown on solid LB medium (10 g tryptone, 10 g sodium chloride, 5 g yeast extracts, and 17 g agar per 1 L) were picked to inoculate 5 mL of 2YT medium (16 g tryptone, 5 g sodium chloride, and 10 g yeast extracts per 1 L) containing 2% glycerol, followed by incubating at 30℃ and 250 rpm overnight. For fermentation, the seed culture was then inoculated into the same fresh medium to yield a final volume of 4 mL with an initial optical density at 600 nm (OD_{600}) of 0.1 and was cultivated at 30°C and 250 rpm for 48 h. The fermentation was performed using two-phase culture by overlaying 1 mL decane over 4 mL culture broth (Yang et al., 2016). For induction, isopropyl-β-D-thiogalactoside (IPTG) was initially added into culture broth at a concentration of 0.1 mM.

2.2. Gene cloning and plasmid construction

Genomic DNA used for gene cloning was extracted from *E. coli* MG1655 (ATCC 700926) and *Saccharomyces cerevisiae* (ATCC 200589). *E. coli* DH5α cells were used for the construction of all recombinant plasmids. PCR primers and plasmids used in this study are listed in Table I and Table II, respectively. All enzymes used for recombinant gene cloning and plasmid construction, such as Phusion high-fidelity DNA polymerase, T4 DNA ligase, and restriction enzymes, were purchased from New England Biolabs. Restriction sites for PacI, PmeI, and NotI were introduced into plasmid pSTV28K by PCR using a primer pair of pS-PmXh-F/pS-PmPa-R, which was followed by digestion with PmeI and ligation, resulting in plasmid pSTV28K2. Using a similar method, plasmid pTrc99A4 was derived from pTrc99A by PCR using a primer pair of pT-PmXhNtI-F/pT-PmPa-R to introduce the restriction sites for PacI, PmeI, NotI, and XhoI.

For construction of the recombinant MEP pathway plasmid ME, first, the MEP pathway genes (*dxs*, *ispC*, *ispDF*, *ispE*, *ispG*, and *ispH*) and *idi* were amplified from the genomic DNA of MG1655 with the primer pairs of dxs-SacI-F/dxs-R, ispC-F/ispC-R, ispDF-F/ispDF-R, ispE-F/ispE-PstI-R, ispG-F/ispG-R, ispH-KpnI-F/ispH-R, and idi-F/idi-KpnI-R; second, the resulting DNA fragments were fused into two multi-gene DNA fragments, namely *dxs-ispDF-ispC-idi* and *ispH-ispG-ispE*, by overlap PCR; finally, the two fused DNA fragments were sequentially assembled into pSTV28K2 using the restriction sites of SacI/KpnI and KpnI/PstI. A protoilludene synthesis operon, which was previously constructed by Yang *et al*. (Yang et al., 2016), was inserted into pTrc99A4 using the restriction sites of EcoRI and SalI, resulting in plasmid AO. To create plasmids of AODxs, AOC,

AODF, AOE, AOG, AOH, and AOI, the MEP pathway genes and *idi* were amplified with their corresponding primer sets containing restriction sites of NotI/XhoI (for *dxs* and *ispC*/*DF*/*E*/*G*) and PacI/PmeI (for *ispH* and *idi*), and introduced into the corresponding restriction sites of plasmid AO. The pBBR1MCS-5 vector was modified to remove the restriction sites for SalI and XhoI by digestion with SalI and XhoI followed by self-ligation. The *fldA* and *fpr* genes from *E. coli* MG1655 were amplified by PCR using the primer pairs of fldA-NtIPstI-F/fldA-BmHI-R and fpr-BmHI-F/fpr-XhIXbI-R and were cloned into the modified pBBR1MCS-5 vector using the restriction sites PstI/BamHI and BamHI/XbaI, respectively, resulting in plasmid pBLP. The DNA fragments containing both *fldA* and *fpr* were amplified from pBLP with two different primer pairs. fldA-PacI-F/fpr-PmeI-R and fldA-NtIPstI-F/fpr-XhIXbI-R, and were cloned into plasmids of ME and AOI using restriction sites PacI/PmeI and NotI/XhoI, respectively, to generate plasmids MELP and AOILP. Plasmid ME-LP, which contains an additional *lac* promoter leading to additional expression of *fldA* and *fpr* as compared with the plasmid MELP, was constructed by amplifying the DNA fragment containing *fldA* and *fpr* along with its *lac* promoter region from pBLP with the primer pair of pBlac-PmeI-F/pB-XhoI-R and introducing it into the restriction sites of PmeI/XhoI of plasmid ME. For the construction of plasmids AOIM, AOIU, and AOItP, the *maeB* and *udhA* genes of *E. coli* and *tPosP5* gene of *S. cerevisiae* were amplified from their corresponding genomic DNA with the primer pairs of maeB-PmeI-F/maeB-XhoI-R, udhA-PmeI-F/udhA-XhoI-R, and tPos5P-PmeI-F/tPos5P-NotI-R, respectively, and were introduced into the restriction sites of NotI and XhoI of the plasmid AOI.

2.3. Quantification of protoilludene by gas chromatography

After the two-phase culture, the decane phase was collected by centrifugation at 10,000 × *g* for 15 min to remove cell debris and subsequently subjected to gas chromatography (GC). Most of the protoilludene produced from the recombinant cells was extracted into the decane phase, and only undetectable or trace amounts remained in the cell pellets. Protoilludene was quantified using an Agilent Technologies 7890A Gas Chromatograph equipped with a flame ionization detector (FID) following our previously reported procedure in which 2 μL of each sample were separated on a 19091N-133 HP-INNOWAX column (length, 30 m; internal diameter, 0.25 mm; film thickness, 0.25 μm) (Yang et al., 2016). The oven temperature was initially held at 50°C and sequentially increased at the rates of 20°C/min to 90°C, 15°C/min to 150°C, 20°C/min to 190°C, and 10°C/min to 260°C. Nitrogen was used as the carrier gas with an inlet pressure of 39.0 psi. The temperature of the detector (FID) was maintained at 280ºC.

3. Results and discussion

3.1. Recombinant MEP pathway for production of protoilludene

To evaluate the capacity of an engineered MEP pathway in *E. coli*, a strong isoprenoid synthesis pathway draining IPP/DMAPP should be coupled with the MEP pathway to prevent an intracellular accumulation of toxic IPP/DMAPP. Our previous report showed that a high-copy, plasmid-based protoilludene synthesis operon (AO in Fig. 2A), encoding *E. coli* farnesyl diphosphate synthase (IspA) and *Omphalotus olearius* protoilludene synthase (Omp7) under a strong *trc* promoter, was highly efficient for conversion of IPP/DMAPP to protoilludene in *E. coli* with an exogenous

MVA pathway (Yang et al., 2016). Protoilludene is a valuable sesquiterpene, and its derivatives are known to exert antitumor and antimicrobial activities (Bohnert et al., 2014). Protoilludene is produced in a trace amount from a recombinant *E. coli* harboring only the plasmid AO encoding the protoilludene synthesis operon (data not shown here). This indicates a poor supply of IPP/DMAPP from the native chromosomal MEP pathway for protoilludene synthesis in the cells. In order to augment MEP pathway, all MEP pathway genes (*dxs*, *ispC*, *ispD, ispE*, *ispF*, *ispG*, and *ispH*) and *idi* were assembled together in a single operon under a moderate *lac* promoter in the low-copy plasmid pSTV28K, resulting in the plasmid ME (Fig. 2A). Co-overexpression of ME and AO resulted in protoilludene production of 10.4 mg/L after 48 h of culture (Fig. 2B). Although there was an increase of protoilludene synthesis by the plasmid ME, the production titer was not satisfactory. To determine the bottleneck in the recombinant MEP pathway, each gene of the recombinant ME pathway was cloned after the *omp7* gene in the plasmid AO (Fig. 2A). Protoilludene production was not improved by the additional overexpression of any of the MEP pathway genes except for the *idi* gene (Fig. 2B). The additional overexpression of IDI using the plasmid AOI significantly increased protoilludene production to 67 mg/L after 36 h of culture. There were also significant differences in cell growth between the strain ME/AOI and the other recombinant strains. Cell growth retardation or inhibition was observed in the recombinant strains with no additional overexpression of IDI, which might be due to the toxicity of IPP, accumulated by the imbalance of IPP/DMAPP synthesis from the recombinant MEP pathway and IPP/DMAPP consumption for protoilludene synthesis. Isoprenyl diphosphates such as IPP are known to be toxic to cells when they accumulate (Martin et al., 2003). The

MEP pathway generates IPP and DMAPP in ratio of 5–6:1 (Rohdich et al., 2003), whereas protoilludene synthesis consumes IPP and DMAPP in ratio of 2:1 (Fig. 1A). If the IDI expression from the plasmid ME is not sufficient, the imbalance between the synthesis and consumption ratios of IPP and DMAPP would cause an accumulation of IPP. Therefore, the improved cell growth and protoilludene production of the strain ME/AOI could be ascribed to a balanced metabolism of IPP/DMAPP when IDI, catalyzing a reversible isomerization of IPP to DMAPP at a ratio of 3:7, is sufficiently expressed. An increase of DMAPP through the overexpression of IDI could also be a benefit to FPP synthase (IspA) possessing a higher Km of 50 μM to DMAPP than 4 μM to IPP (Fujisaki et al., 1986). Furthermore, DMAPP would initiate FPP synthesis more as the reaction priming molecule. The additional overexpression of IspG from the plasmid AOG caused a retardation of cell growth, but a lower growth inhibition than those of the additional overexpression of other MEP pathway genes. However, the protoilludene production was negligible, which indicates a reduced synthesis of FPP by unknown mechanism or mutation. The recombinant strains with the additional overexpression of other MEP pathway genes than IDI and IspG presented a sudden growth after 12 or 24 h of culture, also indicating an unknown metabolic shift or mutation due to the imbalance of generation and consumption of IPP/DMAPP.

3.2. Effect of FldA and Fpr on the performance of a recombinant MEP pathway

A large accumulation of MEcPP, the substrate of IspG, has been observed in *E. coli* harboring a recombinant MEP pathway with high gene dosage (Zhou et al., 2012,

Zou et al., 2013). This indicates that IspG is a rate-limiting step of the recombinant MEP pathway, although its gene dosage is sufficient. Thus, a bottleneck is suspected to be present in the reducing system of FldA and Fpr, known to responsible for functioning of Fe-S cluster proteins such as IspG and IspH (Fig. 1B). The effect of the Fpr-FldA reducing system on the MEP pathway was investigated by overexpression of *fldA* and *fpr* in the protoilludene production strains (Fig. 3). Both *fldA* and *fpr* genes were sequentially assembled downstream of the *ispE* gene on the plasmid ME with a *lac* promoter and low copy number, resulting in a plasmid MELP. Recombinant *E. coli* harboring plasmids MELP and AOI produced 115.1 mg/L of protoilludene, which is an increase of 41% compared to *E. coli* ME/AOI. An additional *lac* promoter was introduced in front of the *fldA*/*fpr* genes on the plasmid MELP to make plasmid ME-LP. Production of protoilludene from *E. coli* ME-LP/AOI was significantly increased to 318.8 mg/L, which is 4.7-fold higher than that of *E. coli* ME/AOI (67.4 mg/L). In order to further increase expression of FldA and Fpr, the *fldA*/*fpr* genes were cloned into the plasmid AOI with a strong *trc* promoter and high copy number, resulting in plasmid AOILP. *E. coli* ME/AOILP produced 183.1 mg/L of protoilludene, which was lower than the highest production obtained from *E. coli* ME-LP/AOI. This suggests that the optimal overexpression of FldA and Fpr is a very effective way to enhance the performance of the MEP pathway through activation of IspG/H reactions. Cell growth is also favored with overexpression of FldA and Fpr. Cell growth of *E. coli* ME-LP/AOI was an OD_{600nm} of 18, which is almost 2-fold higher than that (OD_{600nm} of 10) of *E. coli* ME/AOI. The improved cell growth by overexpression of FldA and Fpr is probably due to accelerated electron transfer, which favors aerobic respiration.

3.3. Effect of NADPH regeneration on the performance of a recombinant MEP pathway

Fe-S cluster proteins, such as IspG/H, are reduced by the complex of Fpr-FldA using NADPH as an electron donor. The conversion of 1-deoxy-D-xylulose 5-phosphate (DXP) to 2-C-methyl-D-erythritol 4-phosphate (MEP) is catalyzed by IspC, which is a NADPH-dependent reaction. Three enzymes (IspC, IspG, and IspH) in the MEP pathway require NADPH as the electron donor for their catalytic reaction (Rohdich et al., 2003, Takahashi et al., 1998). Thus, regeneration of NADPH could be another factor determining the performance of the MEP pathway.

The regeneration of NADPH can be enhanced by three strategies: 1) enhancing the reduction of $NADP^+$ to NADPH, 2) phosphorylation of NADH to NADPH, and 3) reducing the undesired waste of NADPH. First, MaeB and UdhA were targeted to enhance the reduction of $NADP^+$ to NADPH. MaeB is a $NADP^+$-dependent malate dehydrogenase converting $NADP^+$ and (*S*)-malate into NADPH and pyruvate in *E. coli* (Bologna et al., 2007, Csonka and Fraenkel, 1977). MaeB was found to be important for the regeneration of NADPH in *E. coli* during growth on two-carbon compounds, such as acetate (Wang et al., 2011). Overexpression of MaeB is thus expected to facilitate regeneration of NADPH and simultaneously drive a carbon flux from the tricarboxylic acid (TCA) cycle to the MEP pathway through the formation of pyruvate (Fig. 1A). UdhA is a cytosolic pyridine nucleotide transhydrogenase catalyzing the reversible conversion of $NADP^+$/NADH to NADPH/NAD^+ in *E. coli* (Schomburg et al., 2013). Overexpression of UdhA has been reported to enhance the availability of NADPH for several NADPH-dependent metabolic pathways in an

engineered *E. coli* (Lee et al., 2010, Sanchez et al., 2006). Plasmids AOIM and AOIU were constructed by inserting *maeB* and *udhA*, respectively, after *idi* in the plasmid AOI (Fig. 4A). In contrast with our expectations, overexpression of MaeB in the strain ME-LP/AOIM also harboring plasmids ME-LP and AOIM resulted in significant decreases in protoilludene production and cell growth (Fig. 4B). In the strain ME-LP/AOIU, protoilludene production and cell growth were not affected by overexpression of UdhA. The negative effect of MaeB overexpression could be due to depletion of (*S*)-malate by its overconsumption through MaeB, causing an imbalance of cellular metabolism including the TCA cycle. Thus, (*S*)-malate was supplemented in the culture broth at a concentration of 0.1 and 1 mM (Fig. 4C). The protoilludene production and cell growth were restored by the supplementation of (*S*)-malate, although there was no increase in protoilludene production or cell growth compared to the strain ME-LP/AOI with no overexpression of MaeB. The lack of effect of the overexpression of UdhA on protoilludene production and cell growth could be ascribed to its reversible reaction characteristics favoring the reaction direction of $NADPH/NAD^+$ to $NADP^+/NADH$ (Sauer et al., 2004).

Second, in order to increase the pool size of $NADP^+/NADPH$, direct phosphorylation of NADH to NADPH was addressed by overexpression of an NADH kinase. This has been successfully applied for NADPH-dependent biotransformation processes in engineered *E. coli* by heterologous overexpression of tPos5p, a NADH kinase of *S. cerevisiae* lacking the mitochondrial targeting sequence (Lee et al., 2013, Strand et al., 2003). The gene encoding tPos5p was cloned after *idi* on the plasmid AOI, resulting in a plasmid AOItP (Fig. 4A). Production of protoilludene was increased to 479.5 mg/L by overexpression of tPos5p in the strain

ME-LP/AOItP (Fig. 4B).

Third, the *yjgB* gene was deleted to reduce the undesired waste of NADPH. YjgB is a non-essential NADPH-dependent aldehyde reductase with broad substrate specificities (Pick et al., 2013). Although the physiological function of YjgB remains unclear, it was found to have significant catalytic activity in *E. coli* (Zhou et al., 2014). Thus, deletion of *yjgB* gene is expected to reduce the waste of NADPH. In the *yjgB* knockout mutant, *E. coli* DH5α*ΔyjgB*, the plasmid combinations of ME-LP/AOI and ME-LP/AOItP produced 424.5 mg/L and 512.7 mg/L of protoilludene, respectively, which are increases of 33.2% and 7.0% compared to those of the same combinations in the parental DH5α strain. Production of protoilludene was increased with the reduced waste of NADPH. Taken together, the performance of MEP pathway significantly depends on the supply of NADPH.

4. Conclusions

We have provided a new insight into the engineering of the MEP pathway with manipulation of its peripheral metabolic systems. When direct manipulations of a target pathway are confronted with limits in increasing the pathway performance, the manipulation of peripheral metabolic systems could be an alternative approach of metabolic engineering. In this study, an entire recombinant MEP pathway was constructed on the plasmid ME, where additional overexpression of IDI from the plasmid AOI was required to balance the ratio of IPP to DMAPP for their generation (5–6:1) and consumption (2:1) through the MEP pathway and protoilludene synthesis, respectively. Overexpression of the redox recycling system composed of FldA and Fpr was also required for optimal function of IspG and IspH. Heterologous

overexpression of the NADH kinase tPos5p and deletion of the non-essential NADPH-dependent aldehyde reductase YjgB was necessary to enhance performance of the MEP pathway with the three NADPH-dependent reactions of IspC, IspG, and IspH. Through the engineering of the peripheral metabolism of the MEP pathway, protoilludene production was increased from 10.4 mg/L to 512.7 mg/L, which is an improvement of 49-fold in the performance of the recombinant MEP pathway.

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Figure legends

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 are 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.

Fig. 2. Recombinant MEP pathway construction and gene dosage optimization for protoilludene production. (A) Schematic diagram of multi-gene operons corresponding to each plasmid. MEP pathway genes (*dxs*, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, and *ispH*) and *idi* were assembled into a low-copy plasmid (pSTV28K; approx. 10 copies) and transcribed under a moderate *lac* promoter. Protoilludene synthesis genes (*ispA* and *omp7*) were assembled into a high-copy plasmid (pTrc99A; approx. 20 copies) and transcribed under a strong *trc* promoter. An additional copy of each MEP pathway gene was added to the protoilludene synthesis operon. (B) Protoilludene production from *E. coli* engineered with the recombinant MEP pathways. Error bars represent the standard deviation of triplicate experiments.

Fig. 3. Effect of *fpr* and *fldA* overexpression on protoilludene production. (A) Schematic diagram of multi-gene operons corresponding to each plasmid. Both *fpr* and *fldA* genes were co-overexpressed under a single/double *lac* promoter or a single *trc* promoter in different plasmids. (B) Protoilludene production from recombinant *E. coli* with overexpression of *fpr* and *fldA*. Results were obtained after 48 h of culture. Error bars represent the standard deviation of triplicate experiments.

Fig. 4. Effect of manipulation of NADPH regeneration on protoilludene production. (A) Schematic diagram of multi-gene operons containing the *maeB*, *udhA*, and *tPos5p* genes. (B) Effect of overexpression of *maeB*, *udhA*, and *tPos5P* genes on protoilludene production and cell growth in wild-type DH5α and a *yjgB* knockout mutant (DH5α Δ*yjgB*). The DH5α strain harboring plasmids ME-LP and AOI was

used as a control. (C) Effect of (*S*)-malate supplementation on protoilludene production and cell growth in the DH5α strain harboring plasmids ME-LP and AOIM. Results of (B) and (C) were obtained after 48 h of culture. Error bars represent the standard deviation of triplicate experiments.

Table 1

Primers used in this study.

Primers	Sequences[a]	References
pS-PmXh-F	GTGTTAgtttaaacCTTctcgagGGCCGTCGTTTTACAACGTC	This study
pS-PmPa-R	GTGTTAgtttaaacTCttaattaaAGTGCCAAGCTTGCATGCC	This study
pT-PmXhNtI-F	AAAGAgtttaaacCTTctcgaggcggccgcGATGAGAGAAGATTTTCAGCCTGATAC	This study
pT-PmPa-R	GAAGgtttaaacTCTttaattaaAAACAGCCAAGCTTGCATGC	This study
dxs-SacI-F	CGgagctcGCGGACTACATCATCCAGC	This study
dxs-R	TTATGCCAGCCAGGCC	This study
ispDF-F	GGCCTGGCTGGCATAACAACAGGTACTATATGGCAACCACTCATTTGG	This study
ispDF-R	CCTCCTTCATTTTGTTGCCTTAATGAGTA	This study
ispC-F	TAAGGCAACAAAATGAAGGAGGAATCTGATGAAGCAACTCACCATTC	This study
ispC-R	TCAGCTTGCGAGACGC	This study
idi-F	GCGTCTCGCAAGCTGAGGAAGGATTAAGGAGGTTAAACATGCAAACGGAACACGTC	This study
idi-KpnI-R	GGggtaccTTATTTAAGCTGGGTAAATGCA	This study
ispH-KpnI-F	GGggtaccTTTAAGGAGGATAGAACATGCAGATCCTGTTGGCCAA	This study
ispH-R	TTAATCGACTTCACGAATATCGAC	This study
ispG-F	GTCGATATTCGTGAAGTCGATTAAAAAAGAAGGAGGATCAGATGCATAACCAGGCTCCAAT	This study
ispG-R	TTATTTTTCAACCTGCTGAA	This study
ispE-F	TTCAGCAGGTTGAAAAATAAGGAGATTACGTatgCGGACACAGTGGC	This study
ispE-PstI-R	AActgcagTTAAAGCATGGCTCTGTGCA	This study
dxs-NotI-F	TAgcggccgcAATAAGTATTAATAGGCCCCTGAT	This study
dxs-XhoI-R	TCGctcgagTTATGCCAGCCAGGCCTTG	This study
ispC-NotI-F	ATAgcggccgcAGGAGGAATCTGATGAA	This study
ispC-XhoI-R	TAGctcgagTCAGCTTGCGAGACGCAT	This study
ispDF-NotI-F	TAgcggccgcACAACAGGTACTATATGGCA	This study
ispDF-XhoI-R	TAGctcgagTCATTTTGTTGCCTTAATGAGT	This study
ispE-NotI-F	TAgcggccgcAAAAATAAGGAGATTACGTATGC	This study
ispE-XhoI-R	TAGctcgagTTAAAGCATGGCTCTGTG	This study
ispG-NotI-F	TAgcggccgcAAAAAGAAGGAGGATCAGATG	This study
ispG-XhoI-R	TAGctcgagTTATTTTTCAACCTGCTGAAC	This study
ispH-PacI-F	CATttaattaaTTTAAGGAGGATAGAACATGCAGATC	This study
ispH-PmeI-R	TCTCATgtttaaacTTAATCGACTTCACGAATATCGACAC	This study
idi-PacI-F	CATttaattaaGAGGAAGGATTAAGGAGGTTAAACATG	This study
idi-PmeI-R	TCTCATgtttaaacTTATTTAAGCTGGGTAAATGCAGATAATCG	This study
fldA-NtIPstI-F	ATAgcggccgcctgcagCTTTATCCGTGGGCAATTT	This study
fldA-BmHI-R	TTggatccTCAGGCATTGAGAATTTCG	This study
fpr-BmHI-F	TTggatccATTTTTTTGTTCGGAGAACG	This study
fpr-XhIXbI-R	TAGctcgagtctagaTTACCAGTAATGCTCCGCTG	This study
fldA-PacI-F	CTCATgtttaaacCTTTATCCGTGGGCAATTTTC	This study
fpr-PmeI-R	TCTCATgtttaaacTTACCAGTAATGCTCCGCTG	This study
pBlac-PmeI-F	AGCCTTgtttaaacTACGCAAACCGCCTCTCC	This study
pB-XhoI-R	AATctcgagGGCCAGTGAGCGCGCGTA	This study
maeB-PmeI-F	TCTCATgtttaaacCGTTACGTGAAAGGAACAACC	This study
maeB-XhoI-R	TCActcgagTTACAGCGGTTGGGTTTGCG	This study
udhA-PmeI-F	TCTCATgtttaaacAGAACAGGTAAGCCCTACCA	This study
udhA-XhoI-R	TCActcgagTTAAAACAGGCGGTTTAAACCGT	This study
tPos5P-PmeI-F	TCTCATgtttaaacAAGGAGATATATCAAATGAGTACGTTGGATTCACATTCC	This study
tPos5P-NotI-R	TAgcggccgcTTAATCATTATCAGTCTGTCTCTTG	This study
yjgB-KO-F	CTGCCATGCTCTACACTTCCCAAACAACACCAGAGAAGGACCAAAAAATGATTCCGGGGATCCGTCGACC	(Zhou et al., 2014)
yjgB-KO-R	GCGCCTCAGATCAGCGCTGCGAATGATTTTCAAAAATCGGCTTTCA	(Zhou et al.,

ACACTGTAGGCTGGAGCTGCTTCG	2014)

[a] Overlapped nucleotide sequences for fusion PCR are underlined. Restriction sites are presented in lowercase.

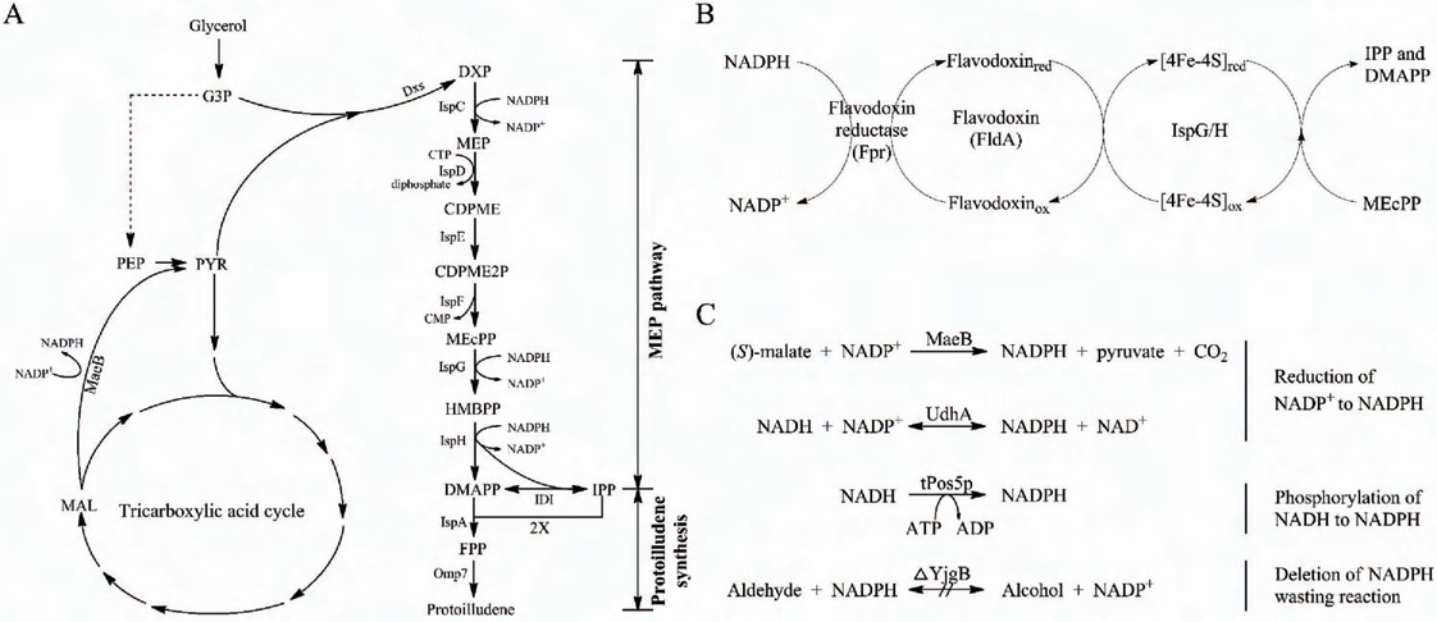
Table 2

Plasmids used in this study.

Plasmids	Descriptions	References
pSTV28K	Derived from pSTV28; P_{lac} cloning vector, pACYC184 origin, *LacZ*, Km^r (Takara Bio Inc., Otsu, Japan)	(Zhou et al., 2014)
pSTV28K2	Derived from pSTV28K; its multiple cloning site contains unique sites for *Eco*R I, *Sac* I, *Kpn* I, *Bam*H I, *Sal* I, *Pst* I, *Sbf* I, *Sph* I, *Hind* III, *Pac* I, *Pme* I and *Not* I in a sequential order	(Amann et al., 1988)
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	This study
pTrc99A4	Derived from pTrc99A; its multiple cloning site contains unique sites for *Nco* I, *Eco*R I, *Sac* I, *Kpn* I, *Sma* I, *Bam*H I, *Xba* I, *Sal* I, *Pst* I, *Hind* III, *Pac* I, *Pme* I, *Not* I and *Xho* I in a sequential order	This study
pBBR1MCS-5	A broad-host-range cloning vector, P_{lac} expression, Gm^r	(Kovach et al., 1995)
AO	pTrc99A4 containing *ispA* from *E. coli* MG1655 and *Omp7* from *Omphalotus olerius*	This study
ME	pSTV28K2 containing *dxs*, *ispDF*, *ispC*, *idi*, *ispH*, *ispG* and *ispE* from MG1655 in a sequential order	This study
pBLP	pBBR1MCS-5 containing *fldA* and *fpr* in a sequential order	This study
AODxs	AO containing *dxs*	This study
AOC	AO containing *ispC*	This study
AODF	AO containing *ispDF*	This study
AOE	AO containing *ispE*	This study
AOG	AO containing *ispG*	This study
AOH	AO containing *ispH*	This study
AOI	AO containing *idi*	This study
pBLP	pBBR1MCS-5 containing *fldA* and *fpr*	This study
MELP	ME containing *fldA* and *fpr* from MG1655 in a sequential order	This study
ME-LP	An additional moderate *lac* promoter was inserted between *ispE* and *fldA* in MELP	This study
AOILP	AOI containing *fldA* and *fpr* in a sequential order	This study
AOIM	AOI containing *maeB* from MG1655	This study
AOIU	AOI containing *udhA* from MG1655	This study
AOItP	AOI containing *tPos5p*, a truncated form of *Pos5p* from *S. cerevisiae*	This study

Highlights

- A new insight into the engineering of the MEP pathway is provided with manipulation of its peripheral metabolic systems.
- Overexpression of the redox recycling system composed of FldA and Fpr is required for optimal function of IspG and IspH.
- Heterologous overexpression of the NADH kinase tPos5p and deletion of the non-essential NADPH-dependent aldehyde reductase YjgB enhance performance of the MEP pathway.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Reduction of NADP+ to NADPH
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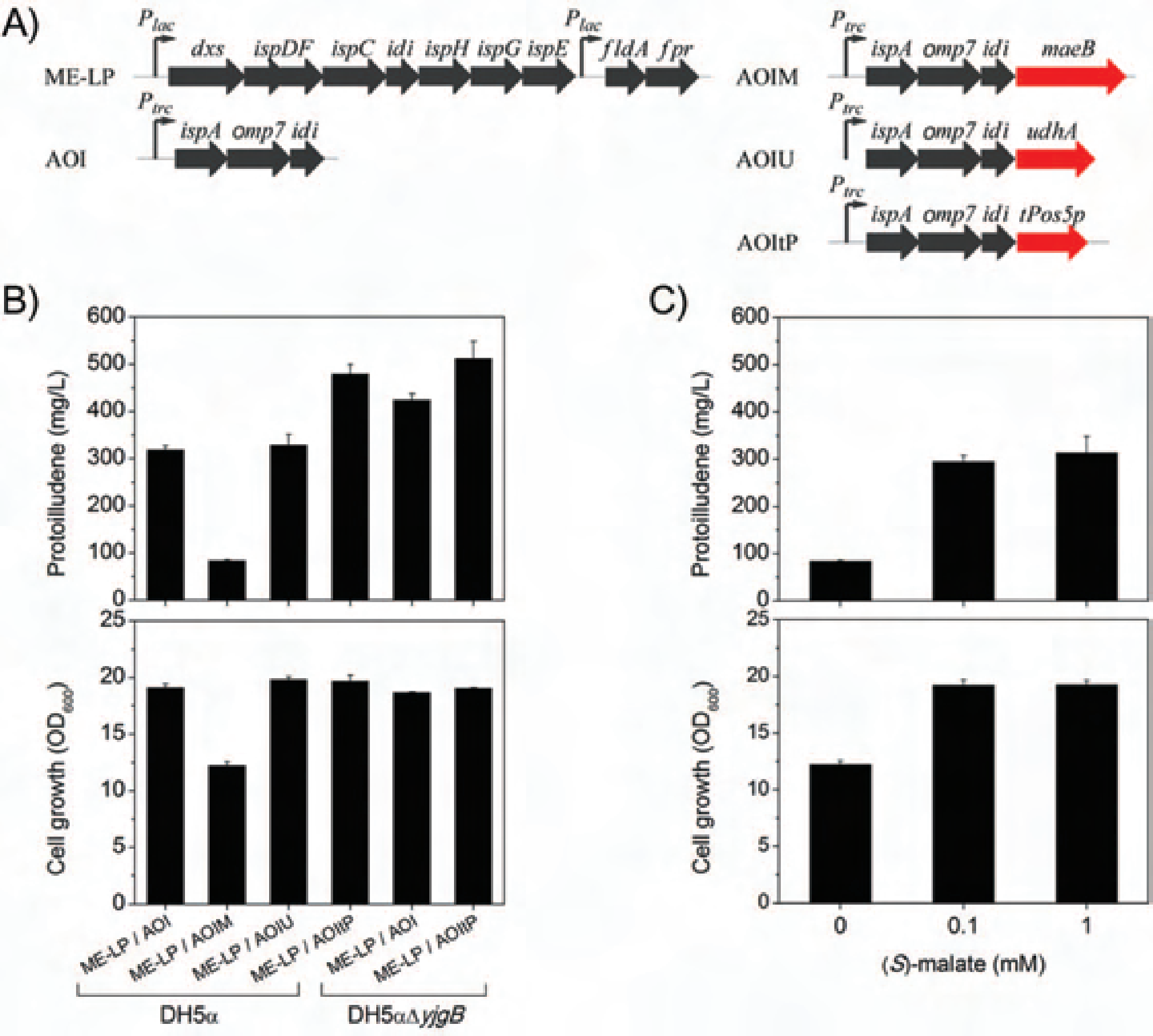
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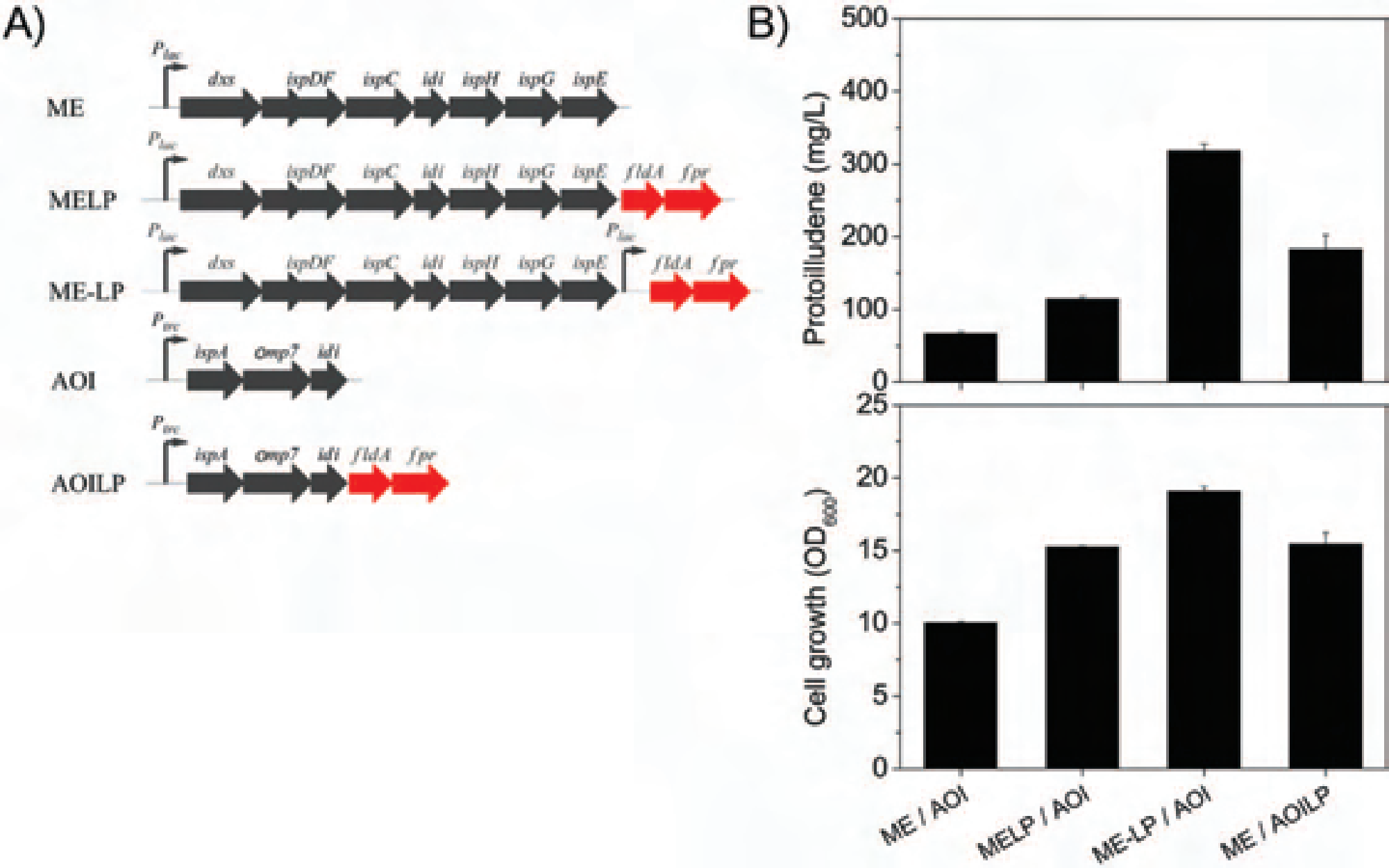
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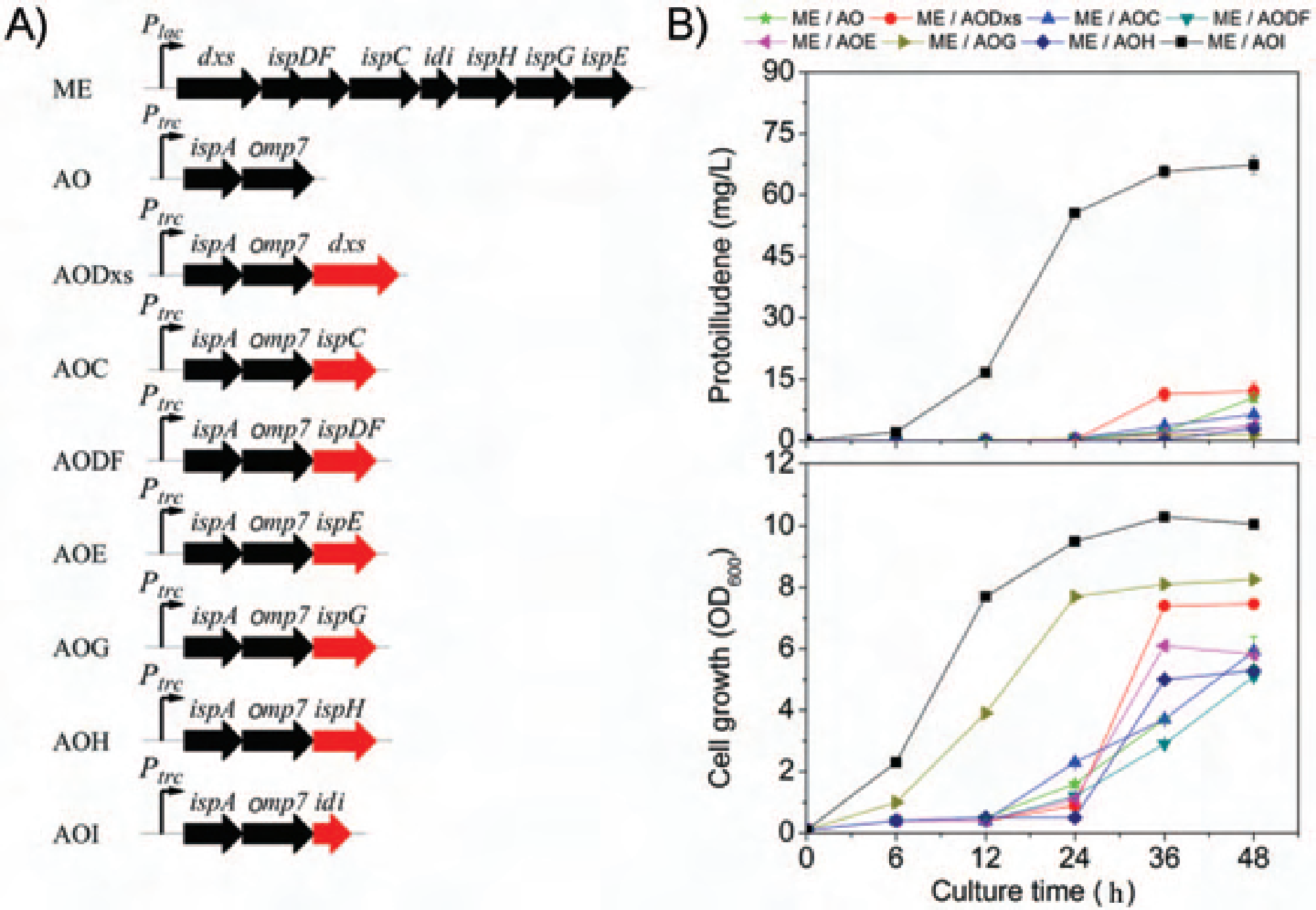
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