Combinatorial engineering of hybrid mevalonate pathways in Escherichia coli for protoilludene production

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Abstract

Background: Protoilludene is a valuable sesquiterpene and serves as a precursor for several medicinal compounds and antimicrobial chemicals. It can be synthesized by heterologous expression of protoilludene synthase in Escherichia coli with overexpression of mevalonate (MVA) or methylerythritol-phosphate (MEP) pathway, and farnesyl diphosphate (FPP) synthase. Here, we present E. coli as a cell factory for protoilludene production.

Results: Protoilludene was successfully produced in E. coli by overexpression of a hybrid exogenous MVA pathway, endogenous FPP synthase (IspA), and protoilludene synthase (OMP7) of Omphalotus olearius. For improving protoilludene production, the MVA pathway was engineered to increase synthesis of building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by sequential order permutation of the lower MVA portion (MvL), the alteration of promoters and copy numbers for the upper MVA portion (MvU), and the coordination of both portions, resulting in an efficient entire MVA pathway. To reduce the accumulation of mevalonate observed in the culture broth due to lower efficiency of the MvL than the MvU, the MvL was further engineered by homolog substitution with the corresponding genes from Staphylococcus aureus. Finally, the highest protoilludene production of 1199 mg/L was obtained from recombinant E. coli harboring the optimized hybrid MVA pathway in a test tube culture.

Conclusions: This is the first report of microbial synthesis of protoilludene by using an engineered E. coli strain. The protoilludene production was increased by approx. Thousandfold from an initial titer of 1.14 mg/L. The strategies of both the sequential order permutation and homolog substitution could provide a new perspective of engineering MVA pathway, and be applied to optimization of other metabolic pathways.

Keywords: Protoilludene, Escherichia coli, Mevalonate pathway, Sequential order permutation, Homolog substitution

Background

Protoilludene derivatives, including illudins, marasmanes and melleolides, are known to exert antitumor and antimicrobial activities [1–3]. For example, the most brilliant potential anticancer agent illudin S, which is first isolated from Omphalotus olearius mushroom, has been studied extensively owing to its cytotoxicity to various tumor cell types [4]. These biological properties and medicinal potential have attracted considerable attention since the late 1960s. Illudins, marasmanes and melleolides can be synthesized from protoilludene by different oxygenation reactions. For example, P450 monooxygenases for the biosynthesis of illudin have been identified from O. olearius [5]. However, protoilludene is naturally produced in a small quantity and its purification from biological material suffers from low yields. Hence, metabolic engineering of microorganisms, such as Escherichia coli, is an alternative and attractive route for the production of protoilludene.

Protoilludene biosynthesis begins with the formation of the universal precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be generated...
via the methylerythritol-phosphate (MEP) pathway and the mevalonate (MVA) pathway [6]. Isopentenyl pyrophosphate and dimethylallyl pyrophosphate are condensed to form farnesyl diphosphate (FPP) by FPP synthase. Linear FPP undergoes multiple electrophilic cyclizations and rearrangements to generate tricyclic protoilludene with an action of protoilludene synthase, which has been isolated from various species including *O. olearius*, *Armillaria gallica*, and *Stereum hirsutum* [7–9]. *O. olearius* protoilludene synthase (OMP7) exhibits a superior catalytic efficiency (Kcat/Km) of \((13.0 \pm 2.0) \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) among those protoilludene synthases (Additional file 1: Table S1) [8].

The entire protoilludene synthesis pathway via the MVA pathway can be divided into three portions, referred to as “MvU” composed of acetyl-CoA acetyltransferase/3-hydroxy-3-methylglutaryl-CoA reductase (MvaE) and 3-hydroxy-3-methylglutaryl-CoA synthase (MvaS), “MvL” composed of mevalonate kinase (MvaK1), phosphomevalonate kinase (MvaK2), diphosphomevalonate decarboxylase (MvaD) and IPP isomerase (IDI), and “AO” composed of FPP synthase (IspA) and protoilludene synthase (OMP7) (Fig. 1). The MVA pathway has been widely engineered for production of several sesquiterpenes in *E. coli* [6, 10–12]. In this study, MVA pathway was engineered for a balanced expression of MvU and MvL portions to increase protoilludene production. The MvL portion was optimized by sequential permutation of its constituent genes in consideration of transcriptional polarity, a general tendency of lower expression of the genes distant from promoter in a multi-cistronic operon [13]. In the optimized MvL portion by the random sequential permutation, the constituent genes would be arranged in their activities from low to high activities in the operon. The expression of MvU portion was modulated by changes of promoters and copy-numbers to tune mevalonate production to its utilization by MvL portion. Optimal coordination of the MvUs and MvLs portions of the MVA pathway were finally able to increase protoilludene production from 1.14 to 721 mg/L. As accumulation of mevalonate intermediate was observed in the culture broth, MvL portion was further engineered by substituting its constituent genes with their homologues from *Staphylococcus aureus*. By the homolog substitution, protoilludene production was increased from 721 to 1199 mg/L in a test tube culture. The successful production of protoilludene from *E. coli* is shown in this work and the recombinant *E. coli* harboring the combinatorially engineered hybrid MVA pathway can serve as a basic platform host for production of other valuable terpenoids.

![Fig. 1 Schematic diagram of protoilludene biosynthesis via mevalonate (MVA) pathway. Protoilludene biosynthesis pathway is divided into three portions: MvU (acetyl-CoA to mevalonate), MvL (mevalonate to IPP and DMAPP) and AO (IPP and DMAPP to protoilludene). MvU portion is composed of MvaE (HMG-CoA reductase/acetyl-CoA acetyltransferase) and MvaS (HMG-CoA synthase). MvL portion is comprised of MvaK1 (mevalonate kinase), MvaK2 (phosphomevalonate kinase), MvaD (diphosphomevalonate decarboxylase) and IDI (IPP isomerase). AO portion consists of IspA (FPP synthase) and OMP7 (protoilludene synthase). Illudins, marasmanes and melleolides are protoilludene derivatives. Pathway intermediates for protoilludene synthesis are as follows: A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVAP, phosphomevalonate; MVAPP, diphosphomevalonate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; and FPP, farnesyl diphosphate. Solid and dashed arrows indicate the identified and unidentified reactions, respectively](image)
spectrometer (GC–MS), and corresponded to 1.14 mg/L of protoilludene. The tiny production could be ascribed to an insufficient supply of IPP and DMAPP from the native MEP pathway.

Thus, the protoilludene synthesis plasmid pTAO was co-transformed with plasmid pSNA [14], which encodes a hybrid exogenous MVA pathway for sufficient supply of IPP and DMAPP, into E. coli DH5α, resulting in the strain E. coli AO/NA. Gas chromatographic analysis showed a specific peak with retention time of 5.7 min, which was subsequently confirmed as protoilludene by GC–MS (Fig. 2b). For 48 h of culture, the strain E. coli AO/NA produced 517 mg/L of protoilludene with an undesired accumulation of mevalonate as much as 571 mg/L (Fig. 2c), indicating the suboptimal performance of MVA pathway encoded by pSNA. It is thus required to redesign the MVA pathway, especially the lower portion of the MVA pathway for protoilludene production.

**Optimization of the MvL portion of the MVA pathway by sequential order permutation**

Expression levels of genes in an operon are known to be affected by their position within the operon [13]. If a gene is located at the tail end of the operon, its expression level is generally lower. Thus, relative expression levels of multi-genes in an operon can be affected by the sequential order of genes in the operon. A specific metabolic pathway encoded by an operon can be optimized by positional modulation of the constituent genes in the operon. Such an approach has been successfully applied to optimization of zeaxanthin synthetic pathway in Bacillus subtilis [15]. The MvL portion of pSNA is composed of four genes SnMvaK1, SnMvaK2 and SnMvaD from Streptococcus pneumoniae, and IDI from E. coli [10]. Optimization of the MvL portion was performed by sequential order permutation of three genes SnMvaK1, SnMvaK2 and SnMvaD. The four genes were assembled in a “Bio-brick” [16] fashion to construct six sequential order permuted lower MVA pathway plasmids (pSMvL1–6) based on pSTV28 vector (Fig. 3a). The strains E. coli AO/MvL1–6 resulting from the co-transformation of pSMvL1–6 and pTAO were evaluated for protoilludene production with supplementation of 4 mM (592.6 mg/L) (R, S)-mevalonate (pSMvL1–6) based on pSTV28 vector (Fig. 3b). The prototype production varied with the sequential order permutation in the MvLs. The highest protoilludene production of 137 mg/L was obtained from E. coli AO/MvL2, whereas E. coli AO/MvL4–6 produced low titers of protoilludene below 25 mg/L. Around 80 mg/L of protoilludene was produced from E. coli AO/MvL3. Residual amounts of mevalonate in the culture broth were measured at the end of the culture to observe the consumption by the strains harboring these sequential order permuted plasmids (Additional file 1: Fig. S2). As expected, the mevalonate consumption generally corresponded to the protoilludene production. The residual mevalonate concentrations in culture of E. coli AO/MvL4–6 were as high as 3 mM (438.5 mg/L), whereas the concentrations in E. coli AO/MvL1, 3 and AO/MvL2 were as low as around 1.7 mM (248.5 mg/L) and 1.3 mM (190.0 mg/L), respectively. Therefore, the lower MVA pathway plasmid pSMvL2 is found to have an optimized gene order for the best performance of the MvL portion, and its order of SnMvaK1–SnMvaD–SnMvaK2–IDI is interestingly consistent with arrangement of the native genes in S. pneumoniae (GenBank: AE007317.1).
Coordination of MvU and MvL portions of MVA pathway for protoilludene production

To optimize the synthesis of mevalonate, the MvU portion of the MVA pathway was cloned into three plasmids with different copy numbers and promoters, pBBR1MCS-2 (6–8 copies and lac promoter), pSTV28 (10–15 copies and lac promoter), and pTrc99A (20–30 copies and trc promoter) [12], which were designated as pBMvUL (LOW), pSMvU M (MEDIUM) and pTMvU H (HIGH), respectively (Fig. 4a). The alternations of copy number and promoter led to the differentiation of mevalonate producing capacity in a range of 10⁴–215 mg/L per OD₆₀₀ at 48 h (Fig. 4b), although there was no significant difference in cell growth among these three strains (Additional file 1: Table S2). In contrast, there was no significant accumulation of mevalonate in the strains of E. coli AO/L1–L6 and E. coli AO/M1–M6, which suggested the lower capacity of the upper portions MvUL and MvUM than the lower portion MvLs (Fig. 4d). In contrast, strains E. coli AO/L1–L6 and E. coli AO/M1–M6 did not exhibit significant mevalonate accumulation (Fig. 4d). However, the poor mevalonate supply from MvUL compared to MvUM seems to restrict the protoilludene production. The highest protoilludene production of 721 mg/L was observed in E. coli AO/M2, which represented a 1.4-fold increase to the production from E. coli AO/NA.

Homolog substitution of the lower MVA portion genes

Kinetic properties of homolog enzymes from different organisms are generally distinct from each other. Homolog enzymes of the lower MVA portion have also different kinetic properties. For example, S. pneumoniae mevalonate kinase (SnMvaK1) is subject to allosteric regulation by diphosphomevalonate, whereas S. aureus mevalonate kinase (SaMvaK1) without the allosteric regulation is competitively inhibited by isoprenyl diphosphates (DMAPP, IPP and FPP) [17, 18]. A metabolic pathway of interest can be improved by substituting a problematic constituent enzyme with its homolog with a desirable property [14]. In order to further improve the mevalonate pathway, the genes of the lower MVA portion MvL2 in pSMvL2 were substituted with their homologs from S. aureus, resulting into a new set of pSMvL7–13 plasmids (Fig. 5a). The effect of the lower MVA portions MvL7–13 on protoilludene production was investigated in combination with the upper MVA pathway portions MvUL and MvUM in the same manner used in Fig. 4c. The upper MVA portion MvUL was excluded in this study because it was suspected to produce insufficient amount of the three plasmids for the combination of MvUs and MvLs in protoilludene production. However, the MvUM plasmid (pSMvUM) is not compatible with pSMvL1–6 derived from the same cloning vector (pSTV28) and MvUM and MvL1–6 are combined in pSMvL1–6-MvUM (Additional file 1: Fig. S4). The MvUL portion was cloned into pTAO plasmid, resulting in pTAO-MvUL, because the MvUL plasmid (pTMvUL) is not compatible with the same vector originated pTAO plasmid (Additional file 1: Fig. S4). Escherichia coli AO/H1–H6 strains harboring pTAO-MvUL and pSMvL1–6 produced a little amount of protoilludene (<35 mg/L; Fig. 4e), accompanying with accumulation of a large amount of mevalonate (>1300 mg/L). It indicated the MvUL produced too much mevalonate beyond the capacity of MvLs and the metabolic unbalance between MvUL and MvLs caused even a significant decrease of cell growth (Additional file 1: Table S2). In contrast, there was no significant accumulation of mevalonate in the strains of E. coli AO/L1–L6 (pTAO/pSMvL1–6/pBMvUL) and E. coli AO/M1–M6 (pTAO/pSMvL1–6-MvUM), which suggested the lower capacity of the upper portions MvUL and MvUM than the lower portion MvLs (Fig. 4d). In contrast, strains E. coli AO/L1–L6 and E. coli AO/M1–M6 did not exhibit significant mevalonate accumulation (Fig. 4d). However, the poor mevalonate supply from MvUL compared to MvUM seems to restrict the protoilludene production. The highest protoilludene production of 721 mg/L was observed in E. coli AO/M2, which represented a 1.4-fold increase to the production from E. coli AO/NA.
of mevalonate for high production of protoilludene. The plasmids pSMvL7-13-MvUM were constructed to combine the upper portion MvUM and the lower portions MvL7–13 (Additional file 1: Fig. S4). The combinations of MvUH and MvL7–13 were conducted by co-transformation of pTAO-MvUH and pSMvL7-13. Interestingly, the strains E. coli AO/M7 (pTAO/pSMvL7-MvUM) and E. coli AO/H7 (pTAO-MvUH and pSMvL7-13), containing the lower MVA portion MvL7 with homolog substitution of SaMvaK1 only, produced the enhanced protoilludene production of 1199 and 740 mg/L, respectively (Fig. 5b and Additional file 1: Table S3). Other homolog substitutions failed to improve production of protoilludene (Fig. 5b). As the homolog substitution of SaMvaK1 with no allosteric inhibition by diphosphomevalonate is effective for protoilludene production, it is suspected the accumulation of diphosphomevalonate in the strain E. coli AO/M2 harboring the lower MVA portion MvL2 due to some bottleneck in the conversion of diphosphomevalonate to IPP by diphosphomevalonate decarboxylase.

**Conclusions**

It is demonstrated the feasibility of producing protoilludene in engineered E. coli. Heterologous expression of the MVA pathway encoded by pSNA enabled the strain E. coli AO/NA to produce 517 mg/L of protoilludene, but mevalonate was accumulated in a significant amount as 571 mg/L due to the unbalanced upper and lower portions of the MVA pathway. To create a balanced efficient MVA pathway, we sequentially permuted the order of genes in the lower portion of the MVA pathway (MvL) and coordinated their expression with the upper portion of the MVA pathway (MvU) by alternations of copy-number and promoter of plasmids. Through this approach, 721 mg/L of protoilludene was produced with reduced accumulation of mevalonate in the strain E. coli AO/M2. The substitution of mevalonate kinase from S. pneumoniae with the homolog from S. aureus further increased protoilludene production to 1199 mg/L. These results suggest that the optimized MVA pathway is efficient to supply IPP and
DMAPP for protoilludene production and also can serve as a platform IPP/DMAPP synthesis pathway for production of other valuable terpenes.

**Methods**

**Bacterial strains and growth conditions**

*Escherichia coli* DH5α were grown in 2YT medium (16 g tryptone, 10 g yeast extract, and 5 g sodium chloride per 1L) at 37 °C for plasmid construction, and at 30 °C for protoilludene production. The seed culture grown overnight at 37 °C was inoculated with an optical density at 600 nm (OD₆₀₀) of 0.1 into 2YT medium containing 2 % (v/v) glycerol. *Escherichia coli* strains (Table 1) harboring the lower portion of the MVA pathway were cultured with addition of 4 mM mevalonate. Ampicillin (100 μg/mL), chloramphenicol (50 μg/mL), kanamycin (50 μg/mL) and 0.2 mM IPTG were added as required. To harvest protoilludene produced during culture, 1 mL of decane was initially overlaid on 4 mL of culture broth. Cell growth was determined by measuring the OD₆₀₀. All experiments were carried out in duplicate.

**Construction of plasmids**

Basic molecular biology procedures, including restriction enzyme digestion and bacterial transformation, were carried out as described in the literature [19]. DNA fragments were amplified by PCR using *Pfu* DNA polymerase (SolGent, Daejeon, Korea) according to the manufacturer’s instructions. BglBricks assembly [16] was applied for

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**Table 1 Strains used in this study**

| Names | Descriptions | Sources |
|-------|--------------|---------|
| *E. coli* AO | *E. coli* DH5α harboring pTAO | This study |
| *E. coli* AO/NA | *E. coli* DH5α harboring pTAO and pSNA | This study |
| *E. coli* AO/MvL1–6 | *E. coli* DH5α harboring pTAO and pSMvL1–6 | This study |
| *E. coli* AO/L1–L6 | *E. coli* DH5α harboring pTAO, pSMvL1–4 and pBMvU | This study |
| *E. coli* AO/M1–M13 | *E. coli* DH5α harboring pTAO and pSMvL1–13–MvUM | This study |
| *E. coli* AO/H1–H13 | *E. coli* DH5α harboring pTAOMvU and pSMvL1–13 | This study |

This table is a brief description of strains used in this study. For more detailed information, refer to Additional file 1: Table 54.

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construction of various plasmids. The schematic diagram of the constructs is shown in figures and the detailed construction process was depicted in Additional file 1. All plasmids and primers used in this study are described in Additional file 1: Table S4.

Identification and quantification of protoilludene

The decane phase of the two-phase culture was collected and centrifuged for 10 min at 12,000 rpm to remove cell debris, and subsequently subjected to gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The production of protoilludene was quantified using an Agilent Technologies 7890A gas chromatograph equipped with a flame ionization detector (FID). One μL of sample was injected at a split ratio of 1:10, and separated on a 19091 N-133 HP-INNOWAX column (length, 30 m; internal diameter, 0.25 mm; film thickness, 250 μm). The oven temperature was initially held at 80 °C for 1 min and was increased at a rate of 10 °C/min to 250 °C, where it was held for 1 min. Nitrogen was used as the carrier gas with an inlet pressure of 39 psi. The detector temperature was maintained at 260 °C. GC-MS analysis was run on a GCMS-2010 ultra mass spectrometer (Shimadzu, Tokyo, Japan). Purified protoilludene was used as the standard compound to construct the standard curve (R² > 0.99) for the estimation of protoilludene production (Additional file 1: Fig. S1).

Quantification of mevalonate

Mevalonate concentration was determined by GC analysis. Culture filtrate was adjusted to pH 2 with 3 M HCl, incubated at 45 °C for 1 h, saturated with anhydrous Na₂SO₄, and extracted with ethyl acetate. The resulting debris, and subsequently subjected to gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The production of protoilludene was quantified using an Agilent Technologies 7890A gas chromatograph equipped with a flame ionization detector (FID). One μL of sample was injected at a split ratio of 1:10, and separated on a 19091 N-133 HP-INNOWAX column (length, 30 m; internal diameter, 0.25 mm; film thickness, 250 μm). The oven temperature was initially held at 80 °C for 1 min and was increased at a rate of 10 °C/min to 250 °C, where it was held for 1 min. Nitrogen was used as the carrier gas with an inlet pressure of 39 psi. The detector temperature was maintained at 260 °C. GC-MS analysis was run on a GCMS-2010 ultra mass spectrometer (Shimadzu, Tokyo, Japan). Purified protoilludene was used as the standard compound to construct the standard curve (R² > 0.99) for the estimation of protoilludene production (Additional file 1: Fig. S1).

Additional file

Additional file 1: Construction of plasmids. Table S1. Comparison of protoilludene synthases reported in literatures. Table S2. Cell growth of recombinant E. coli harboring MVA pathway engineered in a way of various combinations of MvU₄₆ and MvL₁₋₁. Table S3. Cell growth of recombinant E. coli harboring MVA pathway engineered with combinations of MvU₄₆ and MvL₄₋₅. Table S4. Strains, plasmids and primers used in this study. Figure S1. GC-FID standard curve of protoilludene. Figure S2. Residual mevalonate in culture of the strains E. coli AO/MvL₁₋₆ with exogenous addition of mevalonate. Figure S3. Cell growth of E. coli strains harboring pBMvUL, pSMvUM, and pTMvUH. Figure S4. Schematic diagram of pSMvL₁₋₁, MvU₄₆ and pTAQMvUL₁₋₆.
13. Lim HN, Lee Y, Hussein R. Fundamental relationship between operon organization and gene expression. Proc Natl Acad Sci USA. 2011;108:10626–31.
14. Yoon SH, Lee SH, Das A, Ryu HK, Jang JH, Kim HY, Oh DK, Keasling JD, Kim SW. Combinatorial expression of bacterial whole mevalonate pathway for the production of beta-carotene in E. coli. J Biotechnol. 2009;140:218–26.
15. Nishizaki T, Tsuge K, Itaya M, Doi N, Yanagawa H. Metabolic engineering of carotenoid biosynthesis in Escherichia coli by ordered gene assembly in Bacillus subtilis. Appl Environ Microb. 2007;73:1355–61.
16. Anderson JC, Dueber JE, Leguia M, Wu GC, Goler JA, Arkin AP, Keasling JD. BglBricks: a flexible standard for biological part assembly. J Biol Eng. 2010;4:1.
17. Andreassi JL 2nd, Dabovic K, Leyh TS. Streptococcus pneumoniae isoprenoid biosynthesis is downregulated by diphosphomevalonate: an antimicrobial target. Biochemistry. 2004;43:16461–6.
18. Voynova NE, Rios SE, Miziorko HM. Staphylococcus aureus mevalonate kinase: isolation and characterization of an enzyme of the isoprenoid biosynthetic pathway. J Bacteriol. 2004;186:61–7.
19. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 2001.