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Research Paper

Metabolic engineering of *Escherichia coli* for production of valerenadiene

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ABSTRACT

Valeriana officinalis is a medicinal herb which produces a suite of compounds in its root tissue useful for treatment of anxiety and insomnia. The sesquiterpene components of the root extract, valerenic acid and valerena-1,10-diene, are thought to contribute to most of the observed anxiolytic of Valerian root preparations. However, valerenic acid and its biosynthetic intermediates are only produced in low quantities in the roots of *V. officinalis*. Thus, in this report, *Escherichia coli* was metabolically engineered to produce substantial quantities of valerena-1,10-diene in shake flask fermentations with decane overlay. Expression of the wildtype valerenadiene synthase gene (pZE-*wvds*) resulted in production of 12 µg/mL in LB cultures using endogenous FPP metabolism. Expression of a codon-optimized version of the valerenadiene synthase gene (pZE-*cvds*) resulted in 3-fold higher titers of valerenadiene (32 µg/mL). Co-expression of pZE-*cvds* with an engineered methyl erythritol phosphate (MEP) pathway improved valerenadiene titers 65-fold to 2.09 mg/L valerenadiene. Optimization of the fermentation medium to include glycerol supplementation enhanced yields by another 5.5-fold (11.0 mg/L valerenadiene). The highest production of valerenadiene resulted from engineering the codon-optimized valerenadiene synthase gene under strong P_{trc} and P_{T7} promoters and via co-expression of an exogenous mevalonate (MVA) pathway. These efforts resulted in an *E. coli* production strain that produced 62.0 mg/L valerenadiene (19.4 mg/L/OD_{600} specific productivity). This *E. coli* production platform will serve as the foundation for the synthesis of novel valerenic acid analogues potentially useful for the treatment of anxiety disorders.

1. Introduction

Valeriana officinalis is a medicinal wild herb indigenous to many habitats, and the root of this plant is used as a nutraceutical preparation (Valerian) that is currently used for the treatment of anxiety and insomnia (Bent et al., 2006). The roots of *V. officinalis* produce a suite of compounds, including valepotriate alkaloids and sesquiterpenes (Bos et al., 1996). Notably, the sesquiterpene components of Valerian root extract are hypothesized to exhibit many of the beneficial anti-anxiety and anti-insomnia effects. Of the sesquiterpenes, valerenic acid is the most potent GABA-A agonist in these extracts, while valerenal, valerenol, and valerena-1,10-diene (valerenadiene) also modulate GABA-A activity to varying extents in zebrafish and mouse models (Del Valle-Mojica and Ortíz, 2012; Takemoto et al., 2014). Most importantly, the anxiolytic effect of valerian has been demonstrated in human clinical studies in recent years (Anderson et al., 2005; Barton et al., 2011).

Valerenic acid in particular has demonstrated nanomolar binding affinity for the GABA-A receptor (Benke et al., 2009). Recently, the putative binding site of valerenic acid has been determined via docking studies and site-directed mutagenesis (Luger et al., 2015). Luger et al. modeled valerenic acid in the GABA-A receptor in a distinct cleft near the β2/3N265 transmembrane residue. The valerenic acid C-12 carboxyl group is predicted to have important hydrogen-bonding interactions with residues β3N265 and β1S265. Furthermore, the C-13, C-14, and C-15 methyl groups of the valerenane skeleton are predicted to have significant hydrophobic interactions within the pocket at residues within this binding pocket (Fig. 1) (Luger et al., 2015). In summation, these observations have renewed interest in development of novel valerenic acid analogues for structure activity relationship studies.

Despite these advances, valerenic acid is produced as a minor constituent in root tissue of *Valeriana officinalis* (0.7–0.9% DW) (Bos et al., 1998). The low production of valerenic acid hinders further attempts at structure activity relationship and biological activity studies. Synthetic routes toward valerenic acid derivatives are also expensive and ecologically unsuitable. Recently, Ricigliano et al. have engineered *V. officinalis* hairy roots for enhanced production of valerenic acid, which lends credence to a metabolic engineering approach for availing these molecules (Ricigliano et al., 2016). Despite this progress, the hairy root system still produces valerenic acid at very lower titers.

As an alternative approach, metabolic engineering of microbial

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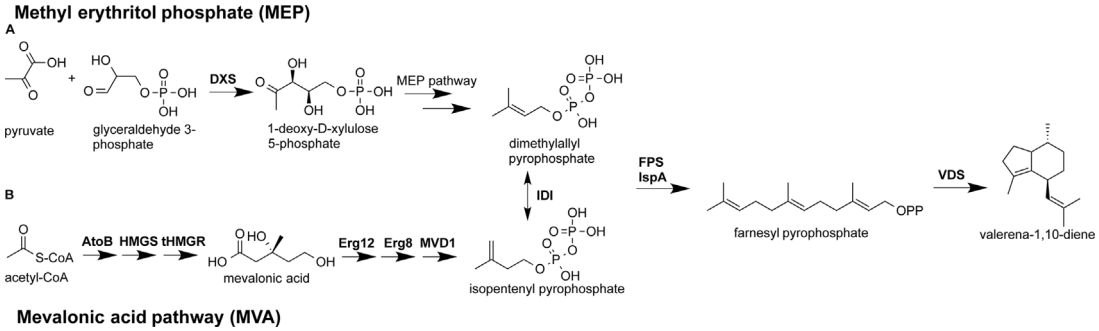

Fig. 1. Biosynthesis of valerenadiene via the methyl erythritol phosphate pathway (MEP, pathway A) and the mevalonic acid pathway (MVA, pathway B). IPP and DMAPP are synthesized via the MEP or MVA pathways via different upstream routes. The MEP pathway is initiated via condensation of glyceraldehyde 3-phosphate and pyruvate from glycolysis via 1-deoxy-D-xylulose 5-phosphate synthase (DXS), and the MVA pathway is initiated via condensation of two molecules of acetyl-CoA. Two molecules of IPP and one molecule of DMAPP are condensed into C_{15} farnesyl pyrophosphate (FPP). FPP is cyclized into valerenadiene via valerenadiene synthase (VDS). The abbreviations of the enzymes are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; atoB, thiolase; HMGS, hydroxy-methylglutaryl-CoA synthase; HMGR, truncated hydroxy-methylglutaryl-CoA reductase; ERG12, mevalonate kinase; ERG8, phosphomevalonate kinase; MVD1, diphosphomevalonate decarboxylase; IDI, isopentenyl diphosphate isomerase; FPS, farnesyl pyrophosphate synthase from *Gallus gallus*; IspA, farnesyl pyrophosphate synthase from *Escherichia coli*; VDS, valerenadiene synthase.

organisms represents a green, cost-effective approach for large-scale production of sesquiterpene pharmaceuticals (Zhang et al., 2011). For example, *Escherichia coli* is one such model host that affords several advantages over plant-based systems due to its fast growth kinetics and capacity to produce high-value chemicals via fermentation on simple carbon sources (Lee, 1996). Furthermore, *E. coli* boasts considerable genetic tools, including multiple promoters and expression vectors that establish it as an ideal host for metabolic engineering. Additionally, terpenes are attractive molecules for metabolic engineering, due to their use as fragrances, flavors, and advanced biofuels (Peralta-Yahya et al., 2011; Sowden et al., 2005). Subsequently, these observations established *E. coli* as a suitable platform for engineering of valerenic acid in this present study.

Two specialized isoprenoid biosynthetic pathways exist for production of C_5 isoprenyl phosphate precursors, dimethyl allyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Many bacteria, algae, and plant chloroplasts employ the methyl erythritol phosphate pathway (MEP), which fluxes glyceraldehyde-3-phosphate and pyruvate towards IPP and DMAPP (Fig. 1) (Rohmer, 1999). Fungi and non-plant eukaryotes utilize the mevalonate pathway (MVA) to convert acetyl-coenzyme A (acetyl-CoA) to IPP and DMAPP via eight enzymatic steps (Fig. 1) (Martin et al., 2003). Subsequently, IPP and DMAPP are concatenated into progressively longer C_{10}, C_{15}, or C_{20} molecules by prenyltransferase enzymes. For sesquiterpene metabolism, farnesyl pyrophosphate synthase condenses 2 IPP units and 1 DMAPP unit to produce farnesyl pyrophosphate (FPP). Subsequently, FPP can be cyclized by a variety of sesquiterpene hydrocarbons by cognate terpene synthases, such as valerenadiene synthase (VDS) (Fig. 1).

However, *E. coli* generates only a finite pool of FPP, and while introduction of a sesquiterpene synthase results in detectable production of sesquiterpenes (Martin et al., 2001), overproduction of the molecule requires redirecting substantial carbon flux to the limited substrate FPP. For example, amorphadiene is a sesquiterpene intermediate in the biosynthesis of the antimalarial drug artemisinin. Martin and co-workers discovered that heterologous expression of the mevalonate isoprenoid pathway from yeast in *E. coli* leads to unregulated carbon flux towards FPP and amorphadiene, which can be semi-synthetically converted to artemisinin (Martin et al., 2003). In using this substrate-engineering approach, Keasling and co-workers have produced amorphadiene in *E. coli* at yields of 500 mg L^{-1} in shake flask (Redding-Johanson et al., 2011) and 27 g L^{-1} in bioreactors (Tsuruta et al., 2009). This generalized approach lends itself to the microbial synthesis of other sesquiterpenes via introduction of variant terpene synthases. To this end, we extended this metabolic engineering concept to the microbial synthesis of valerenadiene. In this report, we developed a metabolic engineering platform for synthesis of valerenadiene in *E. coli* using a codon-optimized valerenadiene synthase and engineered MEP and MVA pathways.

2. Methods and materials

2.1. Bacterial strains and growth conditions

E. coli JM109 (New England Biolabs) was used as the host for all routine cloning manipulations, and *E. coli* DH5αZ1 (Expressys, Germany) and *E. coli* BL21(DE3) (ThermoFisher) were used as hosts for sesquiterpene production (Supplementary Table 1). *E. coli* DH5αZ1 overexpresses a copy of the lacIq repressor on the chromosome for efficient repression of the lac operator (Lutz and Bujard, 1997). Chemically competent *E. coli* were generated with the *E. coli* Mix and Go Transformation Kit (ZYMO Research) and were transformed using standard molecular methodologies (Sambrook and Russell, 2001). *E. coli* strains were grown in LB agar or LB broth at 37 °C for routine maintenance. For production of sesquiterpenes, *E. coli* DH5αZ1 derivatives were grown in 2xYT with supplemented glycerol at 30 °C. for production of valerenadiene. For expression of the mevalonic acid pathway, bacterial growth media was buffered with phosphate buffered saline using a stock solution of 10× PBS (Sambrook and Russell, 2001). Strains were supplemented with ampicillin (100 μg mL^{-1}), chloramphenicol (35 μg mL^{-1}), and kanamycin (50 μg mL^{-1}) as necessary. When multiple plasmids were co-expressed, the chloramphenicol and kanamycin concentrations were adjusted to one-half these amounts.

2.2. Cloning of vds and engineered MEP pathway constructs

Oligonucleotide primers were synthesized by IDT-DNA (Supplementary Table 2), and sequences were verified by sequencing analysis (ACGT, Inc.). Polymerase chain reaction was carried out using Primestar® HS Polymerase (Takara Bio.) by following the manufacturer's protocols. The wildtype valerenadiene synthase gene (*wvds*) gene was amplified via polymerase chain reaction from the pET28a (+)-VoTPS1 (hereafter referred to as pET-*wvds*) construct as described previously (Yeo et al., 2013). Codon usage analysis and codon optimization services were provided via the GenScript OptimumGene™ service before performing gene synthesis. The *cvds* gene was codon-optimized for expression in *E. coli*, synthesized, and spliced into cloning vector pUC57-*cvds* (GenScript). The *cvds* gene was amplified via polymerase chain reaction, and both the *wvds* and *cvds* genes were cut and spliced into the *Eco*RI/*Bam*HI restriction sites of pZE12MCS under the control of the intermediate strength P_{LlacO1} promoter (Expressys, Germany) to afford constructs pZE-*wvds* and pZE-*cvds*, respectively (Lutz and Bujard, 1997). For insertion under the stronger P_{trc} promoter, *cvds* was PCR