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Application of targeted proteomics and biological parts assembly in *E. coli* to optimize the biosynthesis of an anti-malarial drug precursor, amorpha-4,11-diene

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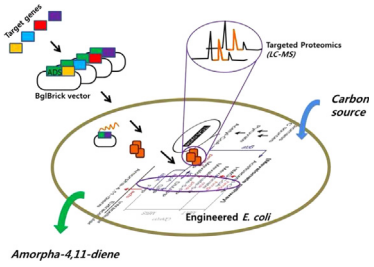
HIGHLIGHTS

- Systematic target gene expressions using BglBrick plasmid are practiced.
- Relatively quantitative protein levels are measured to resolve metabolic bottleneck.
- Combinatorial target gene expression is applied to maximize the final product.

GRAPHICAL ABSTRACT



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ABSTRACT

A balanced heterologous biosynthetic pathway in microbes is necessary to ensure high titers of the desired product. Expression of synthetic heterologous metabolic pathways in a host may not be favorable due to the toxicity of non-native metabolic intermediates and the burden of expression of genes in the pathway. Thus, optimization of gene expression is required to prevent accumulation of the toxic metabolites and to minimize burden. In this study, we used the BglBrick standard cloning vectors to optimize expression of genes in a heterologous mevalonate-based isoprenoid biosynthetic pathway and examined their impact on production of amorpha-4,11-diene, an intermediate in the biosynthesis of the antimalarial drug artemisinin. Amorpha-4,11-diene titer increased almost three-fold when HMG-CoA reductase and phosphomevalonate kinase were overexpressed relative to the original engineered pathway. In addition, selected-reaction monitoring (SRM) mass spectrometry-based targeted proteomics showed that overexpression of HMG-CoA reductase and phosphomevalonate kinase significantly enhances the titer of amorpha-4,11-diene, and that further increases in titer could be achieved if mevalonate kinase were engineered as well.

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1. Introduction

Synthetic metabolic pathways have significant potential for production of pharmaceuticals, specialty and commodity chemicals, and fuels (Keasling, 2010; Nielsen and Keasling, 2011). One of the most important classes of natural products, the isoprenoids,

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has members that are useful in all of these categories. As such, biosynthetic pathways for their production have been engineered into *Escherichia coli* (Ajikumar et al., 2010; Dueber et al., 2009; Jin and Stephanopoulos, 2007; Lee et al., 2008; Martin et al., 2003; Matthews and Wurtzel, 2000; Pfleger et al., 2006; Ro et al., 2006). This is particularly true for the biosynthesis of amorpha-4,11-diene (amorphadiene), a precursor of the antimalarial drug artemisinin, in engineered *E. coli* (Martin et al., 2003; Peralta-Yahya et al., 2011). The production of amorphadiene using a heterologous mevalonate-based isoprenoid biosynthetic pathway in *E. coli* has been studied (Anthony et al., 2009; Ma et al., 2011; Newman et al., 2006; Pitera et al., 2007; Redding-Johanson et al., 2011; Tsuruta et al., 2009). The heterologous pathway consists of five heterologous genes—encoding 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (PMD)—and three native genes—encoding acetyl-CoA thiolase (AtoB), isopentenyl diphosphate isomerase (Idi), and farnesyl pyrophosphate synthase (IspA)—which directs carbon flux from acetyl-CoA to farnesyl pyrophosphate (FPP), the substrate of amorphadiene synthase (ADS) encoded by the synthetic codon-optimized *ads* gene originating from *Artemisia annua* (Fig. 1). Even though there have been a number of studies of this pathway, the highest yield and titer are not yet sufficient for industrial application, most likely due to metabolic imbalances and burden. Determinations of the optimal gene expression for the mevalonate pathway is still needed to increase production (Dueber et al., 2009; Pfleger et al., 2007; Tang and Cirino, 2011; Yoshikuni et al., 2008).

In this work, we further optimized the mevalonate pathway for production of amorpha-4,11-diene. We have used a recently established BglBrick expression vector library (Lee et al., 2011) as a platform for this optimization and used targeted proteomics as a tool to analyze the pathway protein balance (Redding-Johanson et al., 2011). Gene introduction using the BglBrick vector system allowed us to perform gene titration and gene expression studies to determine the effect of gene expression strength on pathway function.

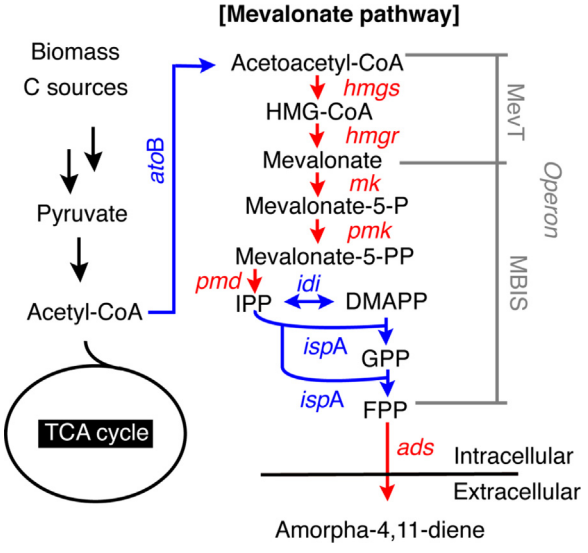

Fig. 1. Metabolic pathway for production of amorpha-4,11-diene from *E. coli* harboring the heterologous mevalonate pathway. The heterologous mevalonate pathway consists of an operon of MevT and MBIS with foreign genes (red color) and native genes (blue color) in an expression plasmid. The MevT portion is composed of *atoB* (acetoacetyl-coA thiolase), *hmgs* (HMG-CoA synthase) and *hmgr* (HMGR-CoA reductase), while the MBIS portion is made up of *mk* (mevalonate kinase), *pmk* (phosphomevalonate kinase), *pmd* (mevalonate diphosphate decarboxylase), *idi* (IPP isomerase) and *ispA* (FPP synthase). *ads* encodes amorpha-4,11-diene synthase. Abbreviations: HMG-CoA, 3-hydroxyl-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used or constructed in this work are listed in Table 1. *Escherichia coli* DH10B and DH1 (Invitrogen, CA) were used for cloning and amorpha-4,11-diene production, respectively. The heterologous mevalonate-based isoprenoid biosynthetic pathway was reconstructed in the plasmid pBbA5c-MevT-MBIS as previously described (Redding-Johanson et al., 2011) (Fig. 1) The MevT operon contains the following genes: *E. coli* thiolase (*atoB*), *S. cerevisiae* HMG-CoA synthase (*HMGS*), and truncated *S. cerevisiae* HMG-CoA reductase (*tHMG1*). The MBIS operon contains the following genes: *S. cerevisiae* mevalonate kinase (*MK*), *S. cerevisiae* phosphomevalonate kinase (*PMK*), *S. cerevisiae* phosphomevalonate decarboxylase (*PMD*), *E. coli* isopentenyl diphosphate isomerase (*idi*), and *E. coli* farnesyl diphosphate synthase (*ispA*). The plasmid pBbE1a-ADS contains a synthetic gene (*ads*) encoding for amorphadiene synthase that converts FPP to amorphadiene.

The strains harboring the mevalonate pathway-containing plasmid and ADS-containing plasmid were grown overnight at 37 °C in LB medium and transferred to fresh EZ-Rich Defined medium (Teknova, Hollister, CA) supplemented with 1% glucose. Production cultures were inoculated at an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.1 and cultivated at 30 °C. Cultures were induced at an OD_{600} of 0.5 with a final concentration of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Samples were collected at 24 h for protein and at 24 h, 48 h, and 72 h for amorpha-4,11-diene measurements. Media were supplemented with 50 μg/mL ampicillin and 35 μg/mL chloramphenicol for plasmid maintenance.

2.2. Assembly of synthetic genes into the BglBrick expression vector library

A library of the BglBrick standard expression plasmids with different origins of replication, antibiotic selection markers, and inducible promoters has been made and their expression characteristics have been documented (Lee et al., 2011). The combination of the replication origins (p15A and ColE1) and promoters (P_{lacUV5} and P_{trc}) in the BglBrick platform plasmids provides different expression strengths of target genes in order to vary the protein level of enzymes in a target pathway (Fig. 2). Each gene of interest in this study was cloned into the standard BglBrick plasmids using BglBrick cloning method to generate the BglBrick single-gene library. Plasmids used or constructed in this work are listed in Table 1. Using the BglBrick cloning method, the *ads* was exchanged for *rfp* on pBbE1a-rfp to construct pBbE1a-ADS, and each gene of interest (X) (i.e. each gene encoding HMGR, MK, and PMK) was added between the *BamHI* and *XhoI* sites of pBbE1a-ADS to construct pBbE1a-ADS-X. DNA sequences of all cloning constructs were verified.

2.3. Amorpha-4,11-diene production assays and targeted proteomics analysis

Amorphadiene titers were analyzed using GC–MS. Samples were prepared and analyzed as described previously (Anthony et al., 2009; Pitera et al., 2007). Extracted amorphadiene was diluted in ethyl acetate (Sigma-Aldrich) spiked with 5 μg/mL (−)-*trans*-caryophyllene as an internal standard. Dodecane/ethyl