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Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene

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ABSTRACT

The introduction or creation of metabolic pathways in microbial hosts has allowed for the production of complex chemicals of therapeutic and industrial importance. However, these pathways rarely function optimally when first introduced into the host organism and can often deleteriously affect host growth, resulting in suboptimal yields of the desired product. Common methods used to improve production from engineered biosynthetic pathways include optimizing codon usage, enhancing production of rate-limiting enzymes, and eliminating the accumulation of toxic intermediates or byproducts to improve cell growth. We have employed these techniques to improve production of amorpha-4,11-diene (amorphadiene), a precursor to the anti-malarial compound artemisinin, by an engineered strain of *Escherichia coli*. First we developed a simple cloning system for expression of the amorphadiene biosynthetic pathway in *E. coli*, which enabled the identification of two rate-limiting enzymes (mevalonate kinase (MK) and amorphadiene synthase (ADS)). By optimizing promoter strength to balance expression of the encoding genes we alleviated two pathway bottlenecks and improved production five fold. When expression of these genes was further increased by modifying plasmid copy numbers, a seven-fold increase in amorphadiene production over that from the original strain was observed. The methods demonstrated here are applicable for identifying and eliminating rate-limiting steps in other constructed biosynthetic pathways.

1. Introduction

In recent years, microorganisms have been engineered to produce compounds of commercial and pharmacological importance. Many of these compounds are either too difficult to synthesize chemically or are produced in limited quantities by their native hosts. Isoprenoids are an example of a class of natural compounds that are of particular interest for microbial production. A number of flavors, fragrances, and medicines are isoprenoids, and most of these compounds are produced in low quantities by their native hosts. In an attempt to produce large quantities of amorpha-4,11-diene (amorphadiene), a precursor to the anti-malarial compound artemisinin, a multi-gene biosynthetic pathway (Fig. 1A) was engineered into *Escherichia coli* (Martin et al., 2003; Newman et al., 2006; Pitera et al., 2007). While the engineered *E. coli* produced relatively high titers of amorphadiene, imbalances in the pathway prevented further improvements in production.

Although engineering microorganisms to produce natural compounds has shown great promise, optimization of these heterologous pathways and host strains is required to obtain maximal titers. Imbalances in gene expression can lead to over- or under-production of enzymes in the pathway, accumulation of toxic metabolic intermediates, and metabolic burden on the host, all of which result in suboptimal product titers (Barbirato et al., 1996; Glick, 1995; Pfleger et al., 2006; Pitera et al., 2007; Zhu et al., 2002). Previously, codon optimization, appropriate promoter and ribosome binding site choice, and directed evolution of enzymes

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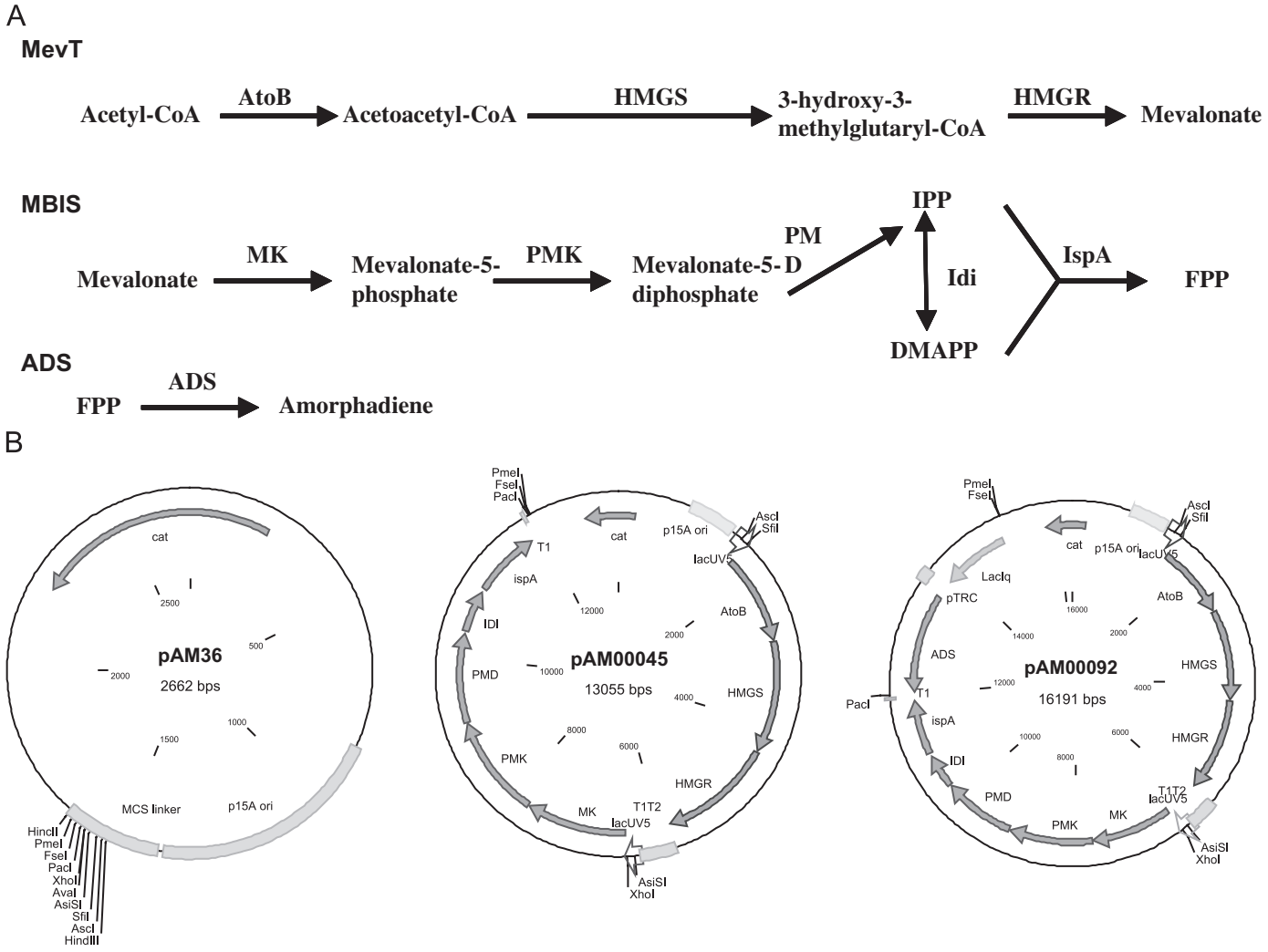

Fig. 1. Mevalonate pathway and vector constructs used in this study. (A) Production of amorphadiene via the mevalonate pathway. The abbreviations for enzymes and pathway intermediates are as follows: AtoB, acetoacetyl-CoA thiolase; HMGS, HMG-CoA synthase; tHMGR, truncated HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate pyrophosphate decarboxylase; Idi, IPP isomerase; IspA, FPP synthase; ADS, amorphadiene synthase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate. (B) Plasmid maps for vectors with multiple cloning site only (pJA4), MevT and MBIS operons (pAM45), or MevT, MBIS, and ADS operons (pAM92). Restriction sites used in cloning are shown, open arrows indicate P_{LacUV5} promoters, and shaded boxes indicate the p15A origin and rrnBT1T2 terminators. The following abbreviations are used: cat, chloramphenicol resistance and $LacI^q$, lac repressor.

have been used to improve production in non-native host strains (Martin et al., 2003; Pfleger et al., 2006; Pitera et al., 2007; Yoshikuni et al., 2006).

This work describes a complimentary method for pathway optimization that focuses on balancing gene expression, identifying rate-limiting enzymes, and decreasing the metabolic burden of the pathway on the host. We sought to improve production of amorphadiene in a previously engineered strain of *E. coli* (Martin et al., 2003). This strain contains plasmid-borne copies of the mevalonate pathway from *Saccharomyces cerevisiae*, as well as the amorphadiene synthase (ADS) gene from *Artemisia annua*. The pathway genes were originally constructed by assembly of the operons onto three different plasmids, each with a different copy number and *E. coli* promoter (Fig. 1A). While this *E. coli* strain proved successful in producing amorphadiene in a two-phase fermentation (~25 mg/L/OD_{600} over 72 h), it did not achieve the desired titers necessary for providing inexpensive artemisinin to people in the Developing World (Newman et al., 2006; Ro et al., 2006). To further optimize expression from this strain, we developed a plasmid system where combinations of various promoters and operon constructs could be easily substituted and tested. Using this system, we identified two rate-limiting enzymes in the pathway and improved production seven-fold over that from the original strain. The strategy presented in this paper of a cloning system with compatible and unique restriction sites for the rapid testing of multiple promoters and operons could be applied readily to any system to identify problems in gene expression and improve production from a heterologous pathway.

2. Materials and methods

2.1. Strains and plasmids

E. coli DH10B was used for cloning. *E. coli* DH1 was used for expression and production studies with the amorphadiene modular vectors. The MevT pathway (operon) contains the following enzymes (genes): acetoacetyl-CoA synthase (*ackA*), HMG-CoA synthases (*HMG*), and an N-terminally truncated version of HMG-CoA reductase (*tHMGR*). The MBIS pathway (operon) contains the following enzymes (genes): mevalonate kinase (*MK*), phosphomevalonate kinase (*PMK*), phosphomevalonate

Table 1
Summary of strains and plasmids used in this study

	Description	Reference
Strain		
DH1	F⁻ *sup*E44 *hsd*R17 *rec*A1 *gyr*A96 *rel*A1 *end*A1 *thi-1* λ-	Hanahan (1983)
DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lacX*74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*) 7697 *gal*U *gal*K λ- *rps*L *nup*G	Invitrogen
Plasmids		
pMevT	pLac33 derivative containing MevT operon; Cm^R	Martin et al. (2003)
pMBIS	pBBR1MCS-3 derivative containing MBIS operon; Tc^R	Martin et al. (2003)
pADS	pTrc99A derivative containing *ADS*; Ap^R	Martin et al. (2003)
pBlueScript IIKS	Cloning vector; Ap^R	Stratagene
pBlueScript-MCS	pBlueScript IIKS derivative containing engineered MCS; Ap^R	This study
pACYC184	Low copy number cloning vector; Cm^R	NEB
pJA4	pACYC184 derivative containing engineered MCS; Cm^R	This study
pAM36-MevT	pJA4 derivative containing codon-optimized MevT operon; Cm^R	This study
pAM43	pAM36-MevT derivative containing MBIS operon; Cm^R	This study
pAM45	pAM43 derivative containing lacUV5 promoters 5′ of the MevT and MBIS operon; Cm^R	This study
pAM45-MevT	pAM45 derivative containing MevT operon; Cm^R	This study
pAM92	pAM45 derivative containing *lacI*^{q}-P_{trc}-*ADS*; Cm^R	This study
pAM94	pADS derivative containing codon-optimized *MK*	This study
pAM29	Cloning vector; p15A origin; *lacUV5* promoter; Kn^R	This study
pAM29-MK	pAM29 derivative containing *MK*	This study
pAM29-PMK	pAM29 derivative containing *PMK*	This study
pAM29-PMD	pAM29 derivative containing *PMD*	This study
pAM29-*idi*	pAM29 derivative containing *idi*	This study
pAM29-*ispA*	pAM29 derivative containing *ispA*	This study

decarboxylase (*PMD*), isopentenyl diphosphate isomerase (*idi*), and farnesyl diphosphate synthase (*ispA*). Plasmids used in this study are listed in Table 1. Media were supplemented with 100 μg/ml carbenicillin, 35 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 10 μg/ml tetracycline to select for plasmid maintenance. All strains were grown at 30 °C.

2.2. Growth conditions

For time-course experiments, overnight cultures were started from fresh transformations into 5 ml of Luria Broth (Difco) with antibiotics. Overnight cultures were diluted to an OD_{600} (optical density at a wavelength of 600 nm) of 0.05 in 40 ml of Terrific Broth (Difco) with 1% glycerol and overlaid with 20% (v/v) dodecane (Sigma-Aldrich). Experiments were initiated when cultures reached an OD_{600} of 0.2–0.3 (~2–3 h after subculture) by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and for the MBIS gene dosage experiments, 20 mM mevalonate (Sigma-Aldrich). Cultures were incubated for 68–75 h, and at each time point the OD_{600} was read and samples from the dodecane phase were taken to determine amorphadiene production. Previous studies have shown that over 97% of the amorphadiene produced partitions into the organic dodecane overlay of cultures (Newman et al., 2006).

2.3. Gas chromatograph-mass selective (GC-MS) analysis of amorphadiene

Samples (100 μl) were removed from the dodecane layer of each culture, and centrifuged at 13,300*g* for 3 min at room temperature. After centrifugation, 10 μl of the organic phase was added to 990 μl of ethyl acetate (Sigma-Aldrich). To measure amorphadiene levels, 1 μl of the sample was injected into an Agilent 6890 series gas chromatograph (GC) equipped with an Agilent 5973 mass selective (MS) detector and a cyclosyl-B (chiral) capillary column (30 m × 250 μm × 0.25 μm thickness, Agilent). The GC oven temperature program was as follows: 100 °C for 45 s, a ramp of 35 °C/min to 200 °C, a ramp of 100 °C/min to 250 °C, and a hold at 250 °C for 1 min. The MS was operated in selected ion monitoring (SIM) mode scanning for the molecular ions at 189 and 204 m/z. Purified amorphadiene was used to generate standard curves for obtaining production titers.

2.4. Construction of a vector for rapid cloning of promoters and amorphadiene operons

An insert containing restriction sites necessary for cloning the amorphadiene pathway was constructed in two pieces by annealing and extending two pairs of oligonucleotides (pACYCfor1, 5′-CAGGAAAGCTTCCGATGGCGCGCCGTAAAGGCCATCCTGGCCTACGCGCGATCGCGGTAGCTCGAGAACGGGTTGACATACG-3′ with pACYCrev1, 5′-CGTATGTCAACCCGTTCTCGAG-3′ and pACYCfor2, 5′-GGTAGCTCGAGAACGGTTAATTAACTCCAGGCCGGCCTACGCGTTTAAACTTCCGGTTGACATACG-3′ with pACYCrev2, 5′-CGTATGTCAACCGGAAGTTTAAAG-3′). Each pair of oligonucleotides (150 pmol) was incubated at 95 °C for 5 min in TE buffer with 50 mM NaCl and 10 mM $MgCl_2$. Reactions were cooled to 25 °C over 20 min. Both products were elongated by adding 10 mM dNTP mix and Klenow DNAP in Buffer 3 (New England Biolabs) and incubating the reactions at 25 °C for 20 min. Fragments were purified via a phenol/chloroform/isoamyl alcohol extraction followed by an isopropanol precipitation. The final products were resuspended in 20 μl TE and digested with *Xho*I. Fragments were ligated to form the multiple cloning site (MCS) insert. Purified ligations were cloned into pBluescript IIKS (digested with *Sma*I, Stratagene) creating pBluescript-MCS. Clones containing the insert were identified via a blue-white screen, and plasmid was purified using Qiagen's Mini-prep kit. The backbone modular vector (pJA4) was constructed by digesting pBluescript-MCS with *Hinc*II and *Hind*III and ligating this insert into a 2.6-kb *Hinc*II/*Hind*II pACYC184 backbone (Fig. 1B).

2.5. Construction of amorphadiene production strains

To increase translation and expression levels in *E. coli*, a codon-optimized MevT operon (MevT66) was synthesized using the *E. coli* codon usage tables. The codon-optimized operon was PCR-amplified with *Sfi*I and *Asi*SI sites and subcloned into the pJA4 vector, resulting in plasmid pAM36-MevT66. The MBIS operon

was PCR-amplified from pMBIS and subcloned into the *Xho*I and *Pac*I sites of pAM36-MevT66, creating pAM43. *LacUV*5 promoters were synthesized from oligonucleotides and subcloned into the *Asc*I/*Sfi*I and *AsiS*I/*Xho*I sites in pAM43, resulting in pAM45 (Fig. 1B). In addition, a non-codon-optimized MevT operon was amplified from pMevT was placed into the *Sfi*I and *AsiS*I sites of pAM45 to create pAM45-MevT. In order to combine the entire amorphadiene pathway into one vector, the *lacI*^{q}-P_{trc}-*ADS* region was PCR-amplified from pADS with *Pac*I and *Fse*I restriction sites, and subcloned into pAM45 to create pAM92 (Fig. 1B).

2.6. Construction of plasmids expressing members of the MBIS operon

To conduct gene dosage experiments, a series of expression vectors containing individual MBIS genes were constructed using the low-copy pAM29 plasmid as a backbone. Plasmid pAM29 was created by assembly of the p15A origin of replication and the kanamycin resistance gene from pZS24-MCS1 (Lutz and Bujard, 1997) with an oligonucleotide-generated *lacUV5* promoter. *MK*, *PMK*, *PMD*, *idi*, and *ispA* were PCR-amplified from pMBIS using primers that added 5′ *Eco*RI 3′*Sal*I restriction sites. The amplified genes were subcloned individually into pAM29 to create the single-gene expression vectors.

Plasmid pMBISopt was created synthetically using DNA synthesis (Codon Devices, Cambridge, MA). It comprises a nucleotide sequence encoding the *S. cerevisiae* melavonate kinase (MK) codon-optimized for expression in *E. coli* (Supplementary Table 1). The nucleotide sequence encoding mevalonate kinase (MK) was PCR-amplified from pMBISopt with *BamH*I and *Hind*III restriction sites, and subcloned into pTrc-ADS to create pAM94.

2.7. Plasmid stability assays

Overnight cultures of DHI pAM92 or DH1 pAM92 pADS were inoculated into 250 ml baffled flasks containing 40 ml of TB+1% glycerol with appropriate antibiotics at a OD_{600} of 0.1. Cultures were incubated with shaking at 220 rpm for a total of 96 h. At 24-, 48-, 72-, and 96-h time points, culture samples were removed and adjusted to OD_{600} of 1.0 with fresh LB medium. Serial dilutions of each culture were plated onto LB and LB with chloramphenicol (for pAM92) and incubated overnight. The number of colonies on each plate were scored and used to calculate viable counts and individual plasmid stabilities.

3. Results

3.1. Increasing expression of the MevT pathway

The design of metabolic pathways can be troublesome because of the large diversity of elements involved in controlling gene expression at both the transcriptional and translational levels: promoter strength, translation efficiency, codon choice, and operon structure. One of the initial modifications made to the original amorphadiene-producing *E. coli* strain was to replace the MevT operon (pMevT, (Martin et al., 2003)) with the *E. coli* codon-optimized operon MevT66. In addition, the wild-type *lac* promoter of pMevT was also replaced with the *lacUV*5 promoter (pAM25), a mutated version of the *lac* promoter whose basal activity is dramatically less sensitive to intracellular levels of cyclic AMP and is ~2-fold stronger than the *lac* promoter (Stefano et al., 1980). These two modifications in DH1 pAM25 pMBIS pADS resulted in an approximate 1.5-fold increase in amorphadiene production at 75 h compared with DH1 pMevT pMBIS pADS (Fig. 2). The

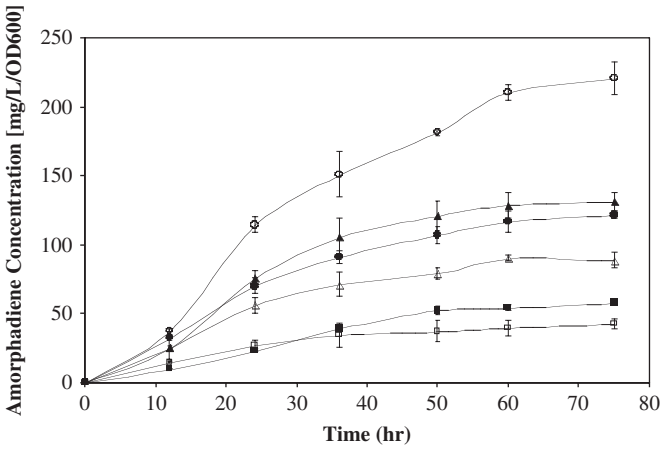

Fig. 2. Decreasing plasmid number and additional copies of ADS increase amorphadiene production. Comparison of amorphadiene production from shake flask cultures of *E. coli* DH1 harboring pMevT pMBIS pADS (□), pAM25 pMBIS pADS (■), pAM45-MevT pADS (●), pAM45 pADS (○), pAM92 (△), and pAM92 pADS (▲). Samples were taken every 12 h for a 72 h period for OD_{600} measurements and amorphadiene titers. Results are the average from three biological replicates with error bars indicating the average standard deviation from the mean.

improvements resulting from optimization of the MevT operon motivated us to examine the use of alternative promoters and gene dosages with the remaining segments of the mevalonate pathway.

3.2. Use of stronger promoters and codon optimization of MevT increases amorphadiene production

To facilitate construction and analysis of mevalonate pathway improvements, we constructed a plasmid expression vector with unique and buffer-compatible restriction sites to efficiently analyze combinations of bacterial promoters and operon constructs. pAM43 served as the initial scaffold for testing potential improvements and contained restriction sites for inserting promoters upstream of the two pathway operons. Due to the previous increase in titers with the *lacUV*5 promoter in pAM25, two *lacUV*5 promoters were inserted into pAM43 to control expression of the mevalonate pathway operons (pAM45-MevT). *E. coli* DH1 harboring pAM45MevT and pADS exhibited a significant increase in amorphadiene production (121 mg/L/OD_{600} at 75 h), which was 3-fold higher than the original base strain, DH1 pMevT pMBIS pADS (Fig. 2). We also tested the codon-optimized MevT66 operon in the context of the vector (pAM45), because of the increase in titer observed previously with pAM25. DH1 pAM45 pADS produced approximately 2-fold more amorphadiene (221 mg/L/OD_{600}) than the non-codon-optimized MevT strain, and a 5-fold increase over the original strain, DH1 pMevT pMBIS pADS. The design of pAM43 with unique and compatible restriction sites enabled the rapid subcloning and testing of other combinations of promoters and operons including the wild-type *lac* promoter for MevT expression, a constitutive promoter driving MBIS expression, and the use of an *E. coli* codon-optimized MBIS operon. However, none of these combinations resulted in a significant increase in amorphadiene production (data not shown).

3.3. Identification of ADS as a limiting enzyme

The presence of multiple plasmids within a bacterial cell can have a profound impact on cellular physiology and often imposes a metabolic burden on the cell (Diaz Ricci and Hernandez, 2000;

Paulsson and Ehrenberg, 1998). This burden can be amplified when multiple plasmids are used for high-level gene expression. It is also thought that expression of plasmid-borne, antibiotic resistance genes necessary for plasmid maintenance also account for a significant portion of the metabolic burden (Rozkov et al., 2004). The original amorphadiene production strain and the derivatives described above contain multiple plasmids and antibiotic resistance genes. This may have created a metabolic burden and compromised product titers. To address this, we placed *ADS* on pAM45 to create a single plasmid with one antibiotic resistance gene. This p15A-replicon plasmid, pAM92, was sufficient for amorphadiene production as it contains the entire mevalonate pathway and ADS gene. Although DH1 harboring pAM92 generated more amorphadiene than the original strain, DH1 pMevT pMBIS pADS, it produced significantly less amorphadiene than the parent strain, DH1 pAM45 pADS (Fig. 2).

Because the mevalonate pathway is identical on both pAM45 and pAM92, we hypothesized that *ADS* levels may limit production. Placing *ADS* on pAM45 lowered the *ADS* copy number from 20–30 copies/cell on pADS (pMB1 origin) to 10–15 copies/cell on pAM92 (p15A origin). Previous studies had also shown that amorphadiene production and enzyme activity dropped to 12% when *ADS* was present on a pBeloBac-11 vector (1 copy/cell) in *E. coli* (C. Paddon, personal communication). In addition, a decrease in cell viability was seen in these strains, presumably due to the accumulation of the intermediate FPP (data not shown). To increase *ADS* expression, we transformed DH1 pAM92 with pADS, resulting in a combined *ADS* gene dosage of 30–45 copies/cell. This strain exhibited an increase in amorphadiene production over DH1 pAM92, but still did not approach the peak titers obtained with DH1 pAM45 pADS (Fig. 2). Experiments to measure pAM92 stability indicated that when this plasmid was expressed alone in DH1 (DH1 pAM92) it was stable ($<10\%$ loss) over a 96 h time course. However, pAM92 in the context of DH1 pAM92 pADS exhibited a decrease in plasmid retention, with an approximate 30% loss over 96 h. These results suggest that the decreased amorphadiene production by DH1 pAM92 pADS was the result of fewer amorphadiene biosynthetic genes due to the loss of pAM92 when these two plasmids were harbored in the same cell.

3.4. Identification of limiting enzymes in the MBIS operon

The primary differences between DH1 pAM25 pMBIS pADS and DH1 pAM45 pADS are the regulation and gene dosage of the MBIS operon (Fig. 1A). In the original pMBIS plasmid, the plasmid backbone was derived from the broad-host range pBBR1-MCS3 plasmid (Kovach et al., 1995, 1994), which is maintained at approximately 6–8 copies per cell. In addition, the MBIS operon is controlled by the catabolite-repressed *lac* promoter. In pAM45, we utilized the *lacUV5* promoter to control MBIS expression and elevated the copy number to 10–15 copies/cell by using the p15A origin of replication rather than the pBBR1 origin. The significant increase in amorphadiene production observed with DH1 pAM45 pADS suggests that one or more enzymes in the MBIS operon is limiting in the original strain.

The results observed with increasing the copy number and promoter strength of the MBIS operon in pAM45 prompted us to perform gene dosage experiments to identify which of the five enzymes in the pathway were limiting. Each of the individual genes in the MBIS operon was PCR-amplified from pMBIS and subcloned into the p15A-based expression vector pAM29. These single-gene expression vectors were transformed into DH1 pMBIS pADS for the gene dosage experiments. Cultures were induced with IPTG and fed 20 mM mevalonate to obtain flux through the pathway. We hypothesized that supplementation of any limiting MBIS gene via the pAM29-derived expression vectors would increase amorphadiene production. This experiment identified *MK*, which encodes mevalonate kinase, the first enzyme in the pathway, as a bottleneck and that additional copies of *MK* increased amorphadiene production 3-fold over the empty vector control (pAM29; Fig. 3). Overexpression of the remaining four genes in the MBIS operon had no significant effect on amorphadiene production (Fig. 3). Finally, transforming additional copies of both *MK* and *PMK*, which encodes phosphomevalonate kinase, resulted in a minimal additional increase in amorphadiene production (data not shown), suggesting that mevalonate kinase was the major bottleneck in the MBIS pathway.

3.5. Combination of modular vectors with increased mevalonate kinase expression

In order to expand upon the increased titers observed when supplementing the pathway with additional mevalonate kinase, we codon-optimized *MK* for use in *E. coli* (Supplementary Table 1) and subcloned the gene 3′ of *ADS* in pADS, creating

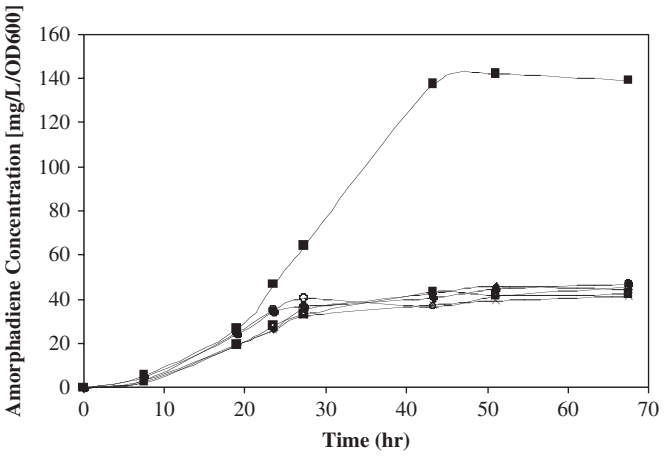

Fig. 3. Mevalonate kinase limits amorphadiene production. Amorphadiene production from shake flask cultures of *E. coli* DH1 pMBIS pADS with an empty vector control (pAM29, ◆) or additional copies of *MK* (■), *PMK* (●), *PMD* (**X**), *idi* (□), or *ispA* (○). Each culture was supplemented with 20 mM mevalonate. Samples were taken over a 68-h period for measurement of OD_{600} and amorphadiene titers.

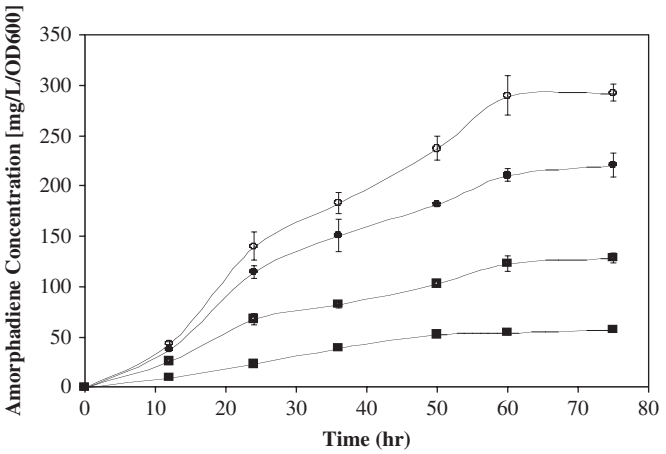

Fig. 4. Amorphadiene production increases when additional copies of mevalonate kinase are present. Amorphadiene production from DH1 harboring pAM25 pMBIS pADS (■), pAM25 pMBIS pAM94 (□), pAM45 pADS (●) or pAM45 pAM94 (○). Samples were taken over a 75-h period and assayed for OD_{600} and amorphadiene titers. Results are the average from three biological replicates with error bars indicating the average standard deviation from the mean.

pAM94. This construct offered the advantage of expressing codon-optimized *MK* from a strong *trc* promoter and a high-copy colE1 origin of replication, maximizing the benefits from *MK* over-expression. pAM94 was tested for amorphadiene production in the context of either the three-plasmid system with DH1 pAM25 pMBIS or in the modular vector system with DH1 pAM45 (Fig. 4). DH1 pAM25 pMBIS pAM94 increased amorphadiene production approximately 2.3-fold over DH1 pAM25 pMBIS pADS. DH1 pAM45 pAM94 had the highest amorphadiene production observed, with a peak specific productivity of 293 mg/L/OD_{600} at 75 h, which represents a seven-fold increase over DH1 pMevT pMBIS pADS.

4. Discussion

Engineering new or modified metabolic pathways into an organism is often a time-consuming and complicated process, especially when the optimal combination of expression elements for a given set of genes is not known. The wide varieties of plasmid copy number, promoter strengths and regulation, ribosome binding sites, and operon structure add to the complexity of successfully generating a high-flux, metabolic pathway. The vectors described in this work, which contain unique cloning sites utilizing compatible restriction enzymes, allowed for the optimization of the amorphadiene biosynthetic pathway. This approach minimizes both the time and effort needed to construct and test many alternative expression constructs. In addition, because multiple operons can be assembled into a single plasmid backbone, the total number of plasmids necessary to contain a complete pathway is reduced, which in turn lessens the metabolic burden on the host.

The goal of our research was to construct plasmid vectors to both analyze pathway bottlenecks and optimize production of amorphadiene, a precursor to the anti-malarial drug artemisinin. Previous work to optimize this pathway involved modulation of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase activity to decrease levels of the toxic intermediate HMG-CoA (Pitera et al., 2007). However, codon optimization of genes and identification of additional limiting enzymes were previously not investigated.

The first improvements in amorphadiene production seen in this study were the result of increased expression from a codon-optimized MevT operon. Our cloning vector construct allowed for rapid screening of promoters, resulting in the replacement of the wild-type *lac* promoters previously used with two-fold stronger *lacUV*5 promoters upstream of both the MevT and MBIS operons. The use of a stronger promoter upstream of MevT resulted in a three-fold increase in production. In addition, the original MevT operon containing two *S. cerevisiae* genes (*HMGS* and *tHMGR*) was replaced with a codon-optimized version in the construct pAM45, resulting in an additional two-fold increase in production. The copy number of the MevT operon remained the same between our original strain and pAM45. However, this was not the case for the MBIS operon, whose copy number increased from 4–6 to 10–15 copies/cell. The increase in production seen from pAM45 suggested that there was a bottleneck in the MBIS pathway. Our experiments identified the first enzyme in the lower half of the pathway, mevalonate kinase, as limiting. By combining the MevT and MBIS operons on our single vector construct we were able to supplement an additional *MK* on a second plasmid to further improve amorphadiene production (DH1 pAM45 pAM94).

Previous studies have shown that the introduction of multi-copy plasmids in *E. coli* and expression of genes from these plasmids results in metabolic burden (Flores et al., 2004; Jones and Keasling, 1998). To maximize metabolic flux through the amorphadiene pathway rather than into plasmid maintenance, we placed the MevT and MBIS operons and *ADS* on a single plasmid, pAM92. Interestingly, DH1 pAM92 did not produce more amorphadiene than DH1 pAM45 pADS. We hypothesized that this decrease in production could be the result of insufficient ADS, because the copy number of this gene was reduced from ~30 to 10–15 copies/cell. To address this issue, we supplemented DH1 pAM92 with pADS, which increased the gene dosage of *ADS* to ~45 copies/cell. DH1 pAM92 pADS produced more amorphadiene than pAM92, suggesting that ADS was limiting, but it still did not reach production levels observed with DH1 pAM45 pADS. Because DH1 pAM92 pADS and DH1 pAM45 pADS have the same mevalonate pathway and plasmid copy number, we suspected that the large size (16.2 kb) of pAM92 might contribute to plasmid instability. However, plasmid stability assays demonstrated that DH1 pAM92 maintained 90% of the plasmid over 96 h suggesting that the plasmid itself was relatively stable. Interestingly, the combination of pAM92 and pADS in the same host resulted in a 30% loss of plasmid over the same period. One hypothesis is that the presence of two strong promoter-gene cassettes (P*trc-ADS*) on two multi-copy plasmids increases the metabolic burden on the cell and results in physiological changes that lead to plasmid loss (Bentley et al., 2004; Ow et al., 2006).

Through the use of these modular vectors, we obtained the highest amorphadiene titers in shake flasks observed thus far (~300 mg/L/OD_{600}). The final strain, DH1 pAM45 pAM94, contained stronger *lacUV*5 promoters, codon-optimized MevT and ADS operons, and additional copies of *MK* and *ADS*. This vector system allowed for quick cloning and analysis of multiple components, resulting in a rapid increase in production over a short period of time. Due to the common methodologies in synthetic biology and pathway construction, we anticipate that this approach should be widely applicable to other metabolic engineering projects.

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.07.007.

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