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Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*

Douglas J. Pitera[a], Chris J. Paddon[b], Jack D. Newman[b], Jay D. Keasling[a,c,d,*]

[a]*Department of Chemical Engineering, University of California Berkeley, CA 94720-1462, USA*
[b]*Amyris Biotechnologies Inc., 5980 Horton Street, Ste. 450, Emeryville, CA 94608, USA*
[c]*Department of Bioengineering, University of California Berkeley, CA 94720-1762, USA*
[d]*Synthetic Biology Department, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*

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Abstract

Engineering biosynthetic pathways in microbes for the production of complex chemicals and pharmaceuticals is an attractive alternative to chemical synthesis. However, in transferring large pathways to alternate hosts and manipulating expression levels, the native regulation of carbon flux through the pathway may be lost leading to imbalances in the pathways. Previously, *Escherichia coli* was engineered to produce large quantities of isoprenoids by creating a mevalonate-based isopentenyl pyrophosphate biosynthetic pathway [Martin, V.J., Pitera, D.J., Withers, S.T., Newman, J.D., Keasling, J.D., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat. Biotechnol. 21, 796–802]. The strain produces high levels of isoprenoids, but upon further investigation we discovered that the accumulation of pathway intermediates limited flux and that high-level expression of the mevalonate pathway enzymes inhibited cell growth. Gene titration studies and metabolite profiling using liquid chromatography–mass spectrometry linked the growth inhibition phenotype with the accumulation of the pathway intermediate 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA). Such an accumulation implies that the activity of HMG-CoA reductase was insufficient to balance flux in the engineered pathway. By modulating HMG-CoA reductase production, we eliminated the pathway bottleneck and increased mevalonate production. These results demonstrate that balancing carbon flux through the heterologous pathway is a key determinant in optimizing isoprenoid biosynthesis in microbial hosts.

Keywords: Pathway engineering; Isoprenoids; Toxicity; Mevalonate pathway; *E. coli*

1. Introduction

Recent trends toward the production of complex chemicals and pharmaceuticals in engineered microbes require continual improvements in the design of non-native biosynthetic pathways. Many potentially useful natural products are either too complex to be chemically synthesized or are produced in insufficient quantities in their native host to implement cost-effective crop cultivation and extraction. Alternatively, through metabolic engineering, multi-gene heterologous pathways have been engineered into microorganisms for the production of many important classes of molecules: isoprenoids (Martin et al., 2003; Watts et al., 2005), polyketides (Pfeifer et al., 2001; Peiru et al., 2005), non-ribosomal peptides (Watts et al., 2005), bioplastics (Aldor and Keasling, 2003), and polymer building blocks (Nakamura and Whited, 2003). However, in the course of transferring enzymatic pathways from one organism to another and modulating protein production, the intricate evolved regulation of the pathway is often lost, leading to imbalances in gene expression and enzyme activity. Overexpression of a gene may cause the depletion of precursors or resources necessary for growth and production (Glick, 1995; Jones et al., 2000) or induce a stress response from excessive heterologous protein

*Corresponding author. Berkeley Center for Synthetic Biology, 717 Potter Street, Building 977, Mail code 3224, University of California, Berkeley, CA 94720-3224, USA. Fax: +1 510 495 2630.
E-mail address: keasling@berkeley.edu (J.D. Keasling).

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(Goff and Goldberg, 1985; Harcum and Bentley, 1993, 1999), while an imbalance in the total activity of the enzymes can restrict carbon flux. Such a bottleneck in the biosynthetic pathway results in a reduced rate of production (Barbirato et al., 1996; Zhu et al., 2002) and can lead to the accumulation of intermediates and byproducts that inhibit pathway enzymes (Berry et al., 2002) or are cytotoxic (Barbirato et al., 1996; Zhu et al., 2001, 2002).

Engineering metabolic pathways in microbes for the production of scarce therapeutic natural products effective against neglected diseases is an attractive application of this technology. For example, to combat the increasing occurrence of malaria-causing *Plasmodium* strains that are resistant to traditional medications, clinicians have employed the potent anti-malarial drug artemisinin, an isoprenoid natural product extracted from *Artemisia annua*. Unfortunately, artemisinin yields from plant extracts are low, limiting production from Artemisia crops and increasing the cost of artemisinin-based treatments beyond the reach of people in countries most afflicted by malaria. For this reason, we engineered the bacterium *Escherichia coli* as a factory for the synthesis of complex isoprenoids such as artemisinin.

One of the largest obstacles to efficient microbial biosynthesis of isoprenoids is the production of isoprenoid precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Both compounds are produced naturally in *E. coli* through the 1-deoxyxylulose-5-phosphate (DXP) pathway (Rohmer et al., 1993; Lange et al., 2000) (Fig. 1A). Metabolic engineering to overproduce the isoprenoid precursors in *E. coli* has focused on optimizing the native DXP pathway and cellular metabolism to accumulate high levels of carotenoids (Farmer and Liao, 2000; Alper et al., 2005b, 2006; Yuan et al., 2006). Combining up-regulation of the entire DXP pathway with systematic and combinatorial gene knockouts resulted in the production of 0.22 g/L lycopene (Alper et al., 2006). This study focuses on an alternative method to over-produce isoprenoid precursors in *E. coli* by cloning and expressing the high flux, mevalonate-dependent, isoprenoid pathway from *Saccharomyces cerevisiae* (Martin et al., 2003) (Fig. 1A). Using the synthesis of a precursor to the potent anti-malarial artemisinin, amorpha-4,11-diene, as an example, this system demonstrated high isoprenoid production capability, producing 0.48 g/L amorphadiene in two-phase fermentation (Newman et al., 2006). This fermentation titer is similar to the aforementioned studies employing the native DXP pathway and represents a 2400-fold improvement in production over wild-type *E. coli*. However, neither production system currently achieves the isoprenoid titer of 25 g/L estimated to be necessary for providing inexpensive artemisinin to countries most afflicted by malaria (Ro et al., 2006).

In the expression of a multi-gene heterologous pathway, the activity of a single enzyme may be out of balance with that of the other enzymes in the pathway, leading to unbalanced carbon flux and the accumulation of an intermediate. In this paper we describe how balancing carbon flux through the heterologous mevalonate pathway resulted in improved growth and isoprenoid production in *E. coli*. This study demonstrates the importance of retaining balanced flux in a reconstituted heterologous pathway and a methodology for troubleshooting and balancing pathways. Whether the molecule is native or foreign to the host, the unregulated accumulation of any intracellular compound may compromise the viability of the organism or the productivity of the pathway.

2. Methods

2.1. Strains and media

E. coli strains TOP10 and DH10B, both from Invitrogen (Carlsbad, CA), were used for cloning and plasmid construction. In *E. coli* DH10B, the P_{BAD} promoter system suffers from all-or-none induction, in which sub-saturating concentrations of arabinose give rise to subpopulations of cells that are fully induced and un-induced (Khlebnikov et al., 2000). To alleviate this problem for gene titration studies, a DH10B host with regulatable control of P_{BAD} in a homogeneous population of cells was constructed. The chromosomal *araE* gene, was placed under constitutive control of the P_{CP8} promoter and combined with a deletion of *araFGH* following the method of Khlebnikov et al. (2001). The resulting strain, which had a linear response in gene expression as a function of the arabinose concentration across the population, was named DP10 (Table 1).

Media components and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). For cloning and propagation of *E. coli* strains harboring the various recombinant pathways, Luria Broth with Miller's modification (Sigma-Aldrich) was used with appropriate antibiotics for plasmid selection and 0.06% glucose for the repression of P_{BAD} and P_{LAC} promoter systems. Microbial production and gene titration studies were conducted in two different versions of C medium (Helmstetter and Cooper, 1968) as described below. D,L-Mevalonate used for media supplementation was prepared by mixing 1 volume of 2 M D,L-mevalonic acid lactone (Sigma-Aldrich) with 1.02 volumes of 2 M KOH and incubating at 37 °C for 30 min (Campos et al., 2001). Enzymes for molecular biology were purchased from New England Biolabs (Beverly, MA) and Invitrogen.

2.2. Operon and plasmid construction

All plasmids used in this study are listed in Table 1. Construction of plasmids pMevT, pMBIS, pADS, pBAD33MevT and pLac33 were described previously (Martin et al., 2003). The MevT operon was cloned into a variety of expression vectors to determine the effect of plasmid copy number and promoter strength on expression of the cloned pathway (Fig. 1B). The MevT operon was previously cloned into pBAD33 (Guzman et al., 1995), a low copy number vector with the arabinose-inducible

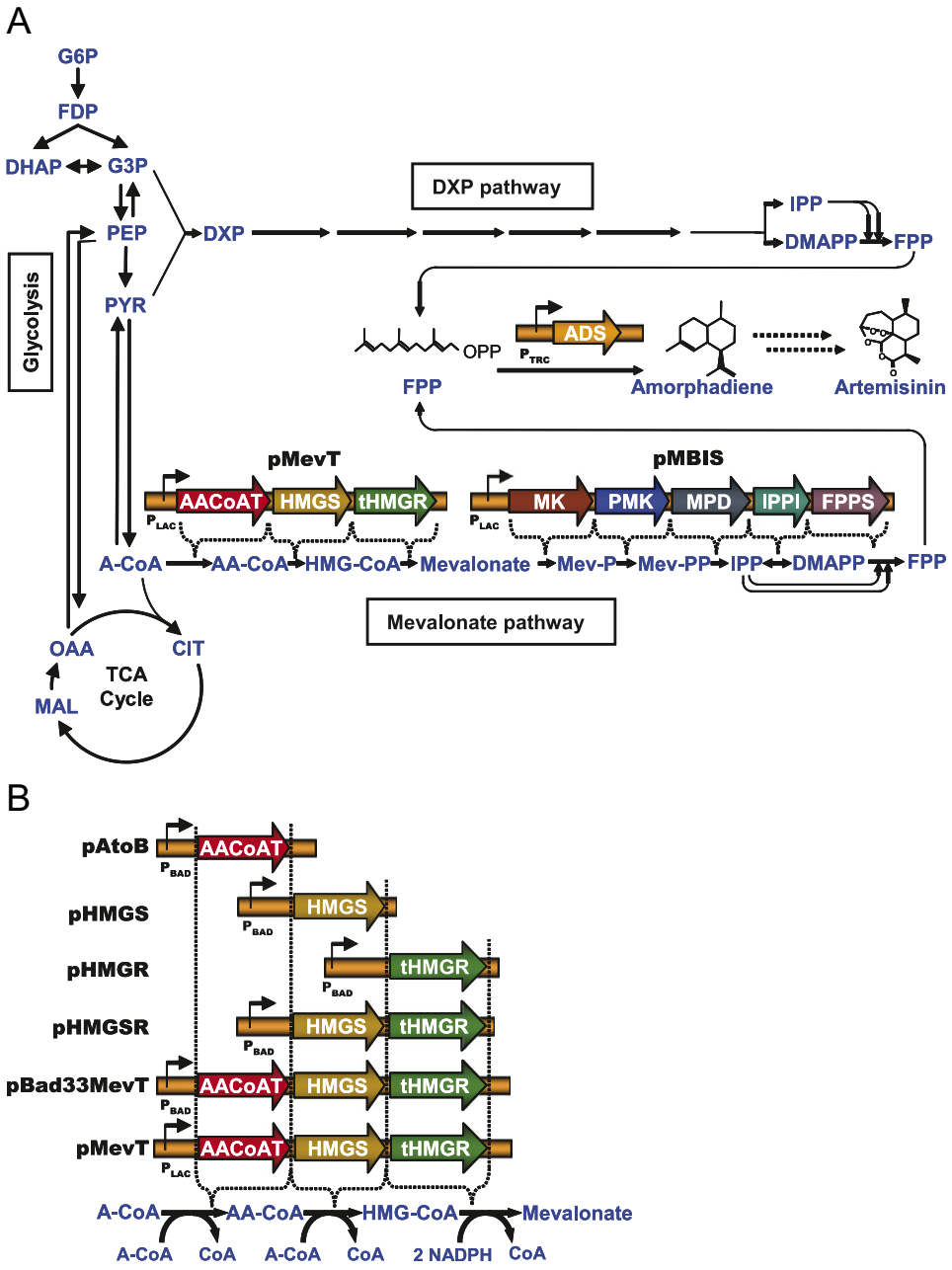


Fig. 1. Mevalonate and DXP pathway to isoprenoid production. (A) Heterologous mevalonate pathway and native DXP (non-mevalonate) pathway for the production of amorphadiene. (B) Mevalonate producing operons and enzyme producing constructs. The abbreviations of the enzymes, the genes encoding the enzymes (in parentheses) and pathway intermediates are as follows: AACoAT, acetoacetyl-CoA thiolase (*atoB*); HMGS, HMG-CoA synthase (*HMGS*); tHMGR, truncated HMG-CoA reductase (truncated *HMG1*); MK, mevalonate kinase (*erg12*); PMK, phosphomevalonate kinase (*erg8*); MPD, mevalonate pyrophosphate decarboxylase (*MVD1*); IPPI, IPP isomerase (*idi*); FPPS, FPP synthase (*ispA*); ADS, amorphadiene synthase (*ADS*); G6P, glucose-6-phosphate; FDP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; PEP, phosphoenolpyruvate; CIT, citrate; MAL, malate; OAA, oxaloacetate; DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; CoA, coenzyme A; A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; Mev-P, mevalonate-5-phosphate; Mev-PP, mevalonate pyrophosphate.

araBAD promoter (P_{BAD}) to create pBAD33MevT (Martin et al., 2003). Expression of the MevT operon was decreased by replacing the *araC*-P_{BAD} fragment of pBAD33MevT with a fragment of pBBR1MCS (Kovach et al., 1995) containing the native *E. coli*, non-consensus, lac promoter (P_{LAC}) to create pMevT (Martin et al., 2003). To increase the copy number of the MevT operon and promoter strength, the three-gene operon was cloned into the *Sal*I site of pBAD24 (Guzman et al., 1995), a medium copy number plasmid containing *araC*-P_{BAD}. The resulting plasmid was named pBAD24MevT.

To investigate sources of growth inhibition, the individual genes that comprise the MevT operon were amplified from pBAD24MevT using standard PCR protocols and

Table 1
Strains and plasmids used in this study

Strain/plasmid	Genotype/description	Source/reference
DH10B	F-, mcrA, Δ(mrr-hsdRMS-mcrBC), F80lacZΔM15, ΔlacX74, recA1, endA1, araD139, Δ (ara, leu)7697, galU, galK, λ-, rpsL (StrR), nupG	Invitrogen
DP10	DH10B, Δ(*araFGH*) ϕ(Δ*araEp* P_{cp8}-*araE*)	This study
pMevT	pBAD33 derivative containing the *atoB*, *HMGS* and *tHMG1* genes under control of P_{LAC}; Cm^r	Martin et al. (2003)
pMBIS	pBBR1MCS-3 containing *erg12*, *erg8*, *MVD1*, *idi*, *ispA* under P_{LAC}; Tc^R	Martin et al. (2003)
pADS	pTrc99A containing synthetic *ADS*; Ap^r	Martin et al. (2003)
pBAD33	Cloning vector containing Cm^r, pACYC184 origin, *araC*, and P_{BAD} promoter	Guzman et al. (1995)
pBAD18	Cloning vector containing Ap^r, modified pBR322 origin with truncation of *rop* gene, *araC*, and P_{BAD} promoter	Guzman et al. (1995)
pBAD24	Cloning vector containing Ap^r, modified pBR322 origin with truncation of *rop* gene, *araC*, and P_{BAD} promoter	Guzman et al. (1995)
pLac33	pBAD33 derivative containing Cm^r, pACYC184 origin, P_{LAC}	Martin et al. (2003)
pTrc99A	Expression vector containing, Ap^r, pBR322 origin, $lacI^Q$, and P_{TRC} promoter	Amann et al. (1988)
pBAD33MevT	pBAD33 containing *atoB*, *HMGS*, *tHMG1* under control of P_{BAD} promoter; Cm^r	Martin et al. (2003)
pBAD24MevT	pBAD24 containing *atoB*, *HMGS*, *tHMG1* under control of P_{BAD} promoter; Ap^r	This study
pAtoB	pBAD33 containing *atoB* under control of P_{BAD} promoter; Cm^r	This study
pHMGS	pBAD33 containing *HMGS* under control of P_{BAD} promoter; Cm^r	This study
pHMGR	pBAD33 containing *tHMG1* under control of P_{BAD} promoter; Cm^r	This study
pHMGSR	pBAD33 containing *HMGS* and *tHMG1* under control of P_{BAD} promoter; Cm^r	This study
pHMGS(C159A)	pHMGS derivative containing *HMGS*(C159A), under control of P_{BAD} promoter; Cm^r	This study
pMevT(C159A)	pBAD33MevT derivative containing *HMGS*(C159A)	This study
pBAD18HMGR	pBAD18 containing *tHMG1* under control of P_{BAD} promoter; Ap^r	This study

inserted into the *Xma*I-*Sal*I sites of the low copy plasmid, pBAD33. *atoB, HMGS,* and *tHMG1,* were amplified using the following primers AtoB-XF, AtoB-SR, HMGS-XF, HMGS-SR, HMGR-XF, and HMGR-SR (see Supplementary Table 1 for oligonucleotide primer sequences). The resulting plasmids containing *atoB, HMGS,* and *tHMG1* alone were named pAtoB, pHMGS, and pHMGR, respectively. To allow co-expression of *tHMG1* with other plasmids, *tHMG1* was amplified as above and cloned into the *Xma*I-*Sal*I sites of the medium copy plasmid, pBAD18 (Guzman et al., 1995), to create pBAD18HMGR.

A two gene operon containing *HMGS* and *tHMG1* was created by amplifying the dual gene segment from pBAD24MevT using primers HMGS-SF and HMGR-SR and inserting the fragment into the *Sal*I site of pBAD33. The resulting plasmid was named pHMGSR.

2.3. Site-directed mutagenesis of HMGS

Following the work of Rokosz et al. (1994), the catalytic cysteine of the *S. cerevisiae* HMGS active site in pBAD33MevT was replaced with an alanine by site-directed mutagenesis using QuickChange Site-directed mutagenesis kit, (Stratagene, La Jolla, CA) with primers mutAHS-F and mutAHS-R, thereby creating a full-length, inactive HMGS. The yeast HMGS mutant with cysteine changed to alanine at amino acid position 159, named HMGS(C159A), was verified by DNA sequence of the entire operon. The plasmid pBAD33MevT containing *HMGS*(C159A) was named pMevT(C159A).

In order to construct a plasmid that expressed mutant HMG-CoA synthase alone, *HMGS*(C159A) was amplified from pMevT(C159A) using primers HMGS-XF and HMGS-SR and cloned into the *Xma*I-*Sal*I sites of pBAD33. The resulting plasmid was named pHMGS(C159A).

2.4. Amorpha-4,11-diene production assays

Amorpha-4,11-diene production from *E. coli* strains expressing the full heterologous mevalonate pathway and amorphadiene synthase with or without mevalonate supplementation was assayed. *E. coli* DH10B containing plasmids pMevT, pMBIS, and pADS (Fig. 1) was grown in C medium (Helmstetter and Cooper, 1968) containing 3.4% glycerol, 1% Casamino acids (BD, Franklin Lakes, NJ), micronutrients, 50 μg/mL carbenicillin, 5 μg/mL tetracycline, and 25 μg/mL chloramphenicol with varying concentrations of exogenous mevalonate. The amorphadiene-producing cells were inoculated from overnight cultures into C medium to an optical density (OD_{600}) of 0.05, incubated at 37 °C with continuous shaking and induced at an OD_{600} of approximately 0.25 with simultaneous addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and varying concentrations of mevalonate. Prior to inoculation, an overlay of 20% (v/v) dodecane (Sigma-Aldrich) was added to each culture flask to trap the volatile sesquiterpene product as described previously (Newman et al., 2006).

The amorphadiene concentration in the organic layer was assayed at multiple time points by diluting 10 μL of the dodecane overlay into 990 μL of ethyl acetate spiked with 5 μg/mL (-)-*trans*-caryophyllene (Sigma) as an internal standard. Dodecane/ethyl acetate extracts were analyzed using an Agilent Technologies (Santa Clara, CA) 6890 gas chromatograph coupled to a 5973 mass selective detector

(GC–MS) as previously described (Martin et al., 2003) by monitoring the molecular ion (204 m/z) and the 189 m/z fragment ion. As amorpha-4,11-diene is not commercially available, amorphadiene concentrations were converted to caryophyllene equivalents using a caryophyllene standard curve and the relative abundance of ions 189 and 204 m/z to their total ions, as described previously (Martin et al., 2003).

2.5. Growth and metabolite analysis of engineered cells

The growth, production, and metabolite levels of *E. coli* DP10 expressing genes encoding the early steps of the mevalonate pathway and combinations thereof were assayed. To accurately characterize the cell physiology and metabolite levels of the engineered strains, studies were performed using fully defined C medium (Helmstetter and Cooper, 1968) supplemented with 3.4% glycerol, all individual amino acids as per Neidhardt et al. (1974), 4.5 μg/mL thiamine-HCl, micronutrients and appropriate antibiotics. In one- or two-plasmid systems chloramphenicol and carbenicillin were used at 50 μg/mL each. Starter cultures of DP10 harboring various genetic constructs were inoculated from single colonies and incubated overnight at 37 °C in defined C medium supplemented with 0.06% glucose (to repress P_{BAD} and P_{LAC}) and antibiotics. Overnight starter cultures were diluted to an OD_{600} of 0.05 in fresh medium, incubated at 37 °C with continuous shaking and induced with the addition of 1.33 mM (0.02%) arabinose or 0.5 mM IPTG at an OD_{600} of approximately 0.25. When assaying only cell growth, cultures were grown in 96-well microtiter plates. During experiments involving metabolite analysis the engineered strains were incubated in 1-L baffled shake flasks. Samples were taken at multiple times points during the course of the experiment to assay for OD_{600}, mevalonate production, and intracellular metabolites. Experiments were repeated in duplicate to confirm trends in growth and metabolite accumulation.

The optical densities of samples taken from cultures in baffled flasks were measured using a UV–Vis spectrophotometer (Beckman, Fullerton, CA), while the OD_{600} of cultures in 96-well microtiter plates was measured using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

2.6. GC–MS quantification of mevalonate

Mevalonate (mevalonic acid) concentration in cultures of engineered *E. coli* was determined by GC–MS analysis. An aliquot (560 μL) of *E. coli* culture was mixed with 140 μL of 500 mM HCl in a glass GC vial to convert mevalonic acid to mevalonic acid lactone. Ethyl acetate (700 μL), containing 500 μg/mL (-)-*trans*-caryophyllene as an internal standard, was added to each vial and then the samples were shaken at maximum speed on a Fisher Vortex Genie 2 mixer (Fisher Scientific) for 5 min. The ethyl acetate extract of the acidified culture was diluted 1:100 with fresh ethyl acetate in a clean GC vial before analysis.

Diluted ethyl acetate extracts were analyzed using a GC–MS operated in electron impact mode. The GC column used was an Agilent Technologies DB-5 ms (30 m × 250 μm × 0.25 μm). Helium was used as the carrier gas at a constant flow of 1 mL/min and 1 μL split-less injections were performed. The injection port was maintained at 250 °C, the MS source temperature was maintained at 230 °C, and the MS quad temperature was held constant at 150 °C. The column temperature profile was 70 °C for 2 min; 15 °C/min to 185 °C; 30 °C/min to 300 °C; and held at 300 °C for 3 min. The selected ions monitored were m/z 71 and 58 for mevalonic acid lactone, and m/z 189 and 204 for (-)-*trans*-caryophyllene. Retention time, mass spectrum, and concentration of extracted mevalonic acid lactone were confirmed using commercial DL-mevalonic acid lactone (Sigma).

2.7. Intracellular metabolite extraction and analysis

The concentrations of intracellular acyl-CoA and adenylate pools were determined by LC–MS analysis of trichloroacetic acid (TCA) culture extracts taken during the exponential phase of growth. To simultaneously and rapidly quench cellular metabolism, isolate *E. coli* cells from growth medium, and extract metabolites, cells were centrifuged through a layer of silicone oil into a denser solution of TCA similar to previous methods (Shimazu et al., 2004). Using 15-mL Falcon tubes (Fischer Scientific), 2 mL of silicone oil (AR200 from Fluka) was layered over 0.5 mL of 10% TCA (Fluka) in deuterium oxide (D_2O; Sigma-Aldrich). The TCA layer contained crotonyl-CoA as an internal standard. Tubes were stored on ice until the time of sampling. To each tube, 10 mL of cell culture was carefully added above the silicone oil layer. Tubes were then quickly centrifuged at 4 °C for 3 min at 10,000g. The spent medium was carefully removed by aspiration and the TCA extract layer was transferred to a 2 mL centrifuge tube using a small gauge needle and syringe. To neutralize the TCA, 1 mL of ice cold 0.5 M Tri-n-octylamine in 1,1,2-Trichloro-1,2,2-trifluoroethane (both Sigma) was added, tubes were vortexed for 1 min and then centrifuged at max speed for 2 min to separate the layers. The aqueous layer was removed for analysis by LC–MS.

The neutralized TCA extract was analyzed using an Agilant Technologies 1100 series LC–MS using electrospray ionization. A 50 μL sample was separated on a C-18 reverse-phase HPLC column (250 × 2.1 mm Inertsil 3 μm ODS-3 by Varian, Palo Alto, CA) using a gradient of 100 mM ammonium acetate buffer at pH 6 (solvent A) and 30% acetonitrile in 70% solvent A (solvent B). The HPLC column was equilibrated each run with 8% solvent B for 12 min. Using a 0.25 mL/min flow rate and linear gradients as indicated, the elution program was the following: 8% B at 0 min to 50% B at 5 min, gradient increase to 100% B at 13 min, isocratic at 100% B until 19 min, gradient returning

to 8% B at 26 min. The resolved metabolite samples were analyzed using an electrospray ionization mass selective detector (ESI-MS) operated in positive mode. The following ESI-MS parameters were used: drying gas, 12 L/min; nebulizer pressure, 60 psig; drying gas temperature, 300 °C; capillary voltage, 2500 V. Selected ions corresponding to the protonated molecular ion of each metabolite were monitored: adenosine 5′-triphosphate (ATP), *m/z* 508; adenosine 5′-diphosphate (ADP), *m/z* 428; adenosine 5′-monophosphate (AMP), *m/z* 348; coenzyme A, *m/z* 768; acetyl-CoA, *m/z* 810, propionyl-CoA, *m/z* 824; crotonyl-CoA, *m/z* 836; acetoacetyl-CoA, *m/z* 852; malonyl-CoA, *m/z* 854; succinyl-CoA, *m/z* 868; methylmalonyl-CoA, *m/z* 868; and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), *m/z* 912. Retention times, mass spectra, and concentrations of extracted metabolites were confirmed using commercial standards (Sigma-Aldrich). Adenylate energy charge of each strain was calculated from the adenylate pool measurement as defined by Atkinson (1968)

$$\text{Energy Charge (EC)} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}. \quad (1)$$

2.8. HMG-CoA reductase assays

tHMGR activity in *E. coli* DP10 lysates expressing different MevT constructs was assayed by monitoring the disappearance of NADPH at 340 nm (Rodwell et al., 2000). Samples of cell culture were taken at various time points after induction, centrifuged at 10,000*g* for 8 min to pellet the cells, and the supernatant was removed. Cell pellets were frozen on dry ice until analysis. Cells were lysed using Bug Buster reagent (EMD Biosciences, San Diego, CA) supplemented with 40 μg/mL lysozyme, protease inhibitor, and benzonaze, following the manufacturer's protocol. Total protein concentrations of the lysates were determined by using the Bradford protein assay kit (Bio-Rad, Hercules, CA) employing bovine serum albumin as a standard. HMG-CoA reductase assays were performed in 100 μL reactions in microtiter plates at 30 °C. Reaction mixtures consisted of 50 mM Tris–HCl pH 7.5, 0.2 mM NADPH, and 0.3 mM HMG-CoA. Reactions were started with the addition of diluted cell lysate.

3. Results

3.1. Initial steps of heterologous pathway limits carbon flux to amorphadiene

To identify the limiting steps in the heterologous pathway, *E. coli* DH10B cultures expressing the full pathway from acetyl-CoA to amorphadiene (harboring plasmids pMevT, pMBIS, and pADS—Fig. 1A) were grown in medium supplemented with increasing concentrations of exogenous mevalonate. GC–MS analysis revealed that the addition of exogenous mevalonate to the medium

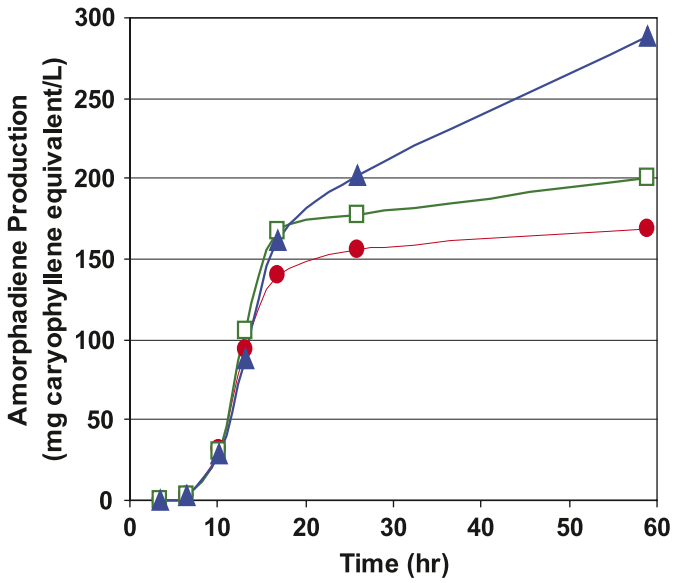


Fig. 2. Amorphadiene production from shake flask cultures of *E. coli* DP10 harboring pMevT, pMBIS, and pADS with either no (●), 10 mM (□), or 20 mM (▲) mevalonate supplemented to the medium.

increased the production of amorphadiene over time, above that produced with no mevalonate supplementation (Fig. 2). Since supplementary mevalonate feeds into the mevalonate pathway at the pMBIS enzymes and is converted to additional amorphadiene (as tested up to the addition of 20 mM D,L-mevalonate), this increase in amorphadiene production implies that the supply of mevalonate by the MevT operon limited flux through the pathway.

The mevalonate pathway begins with acetyl-CoA, an abundant cellular metabolite. Studies on recombinant poly(3-hydroxybutyrate) (PHB) production demonstrated that acetyl-CoA is available in *E. coli* to produce up to 194.1 g/L of PHB at a rate of 4.63 g/L/h from the native supply of acetyl-CoA (Choi et al., 1998; Choi and Lee, 1999). Additionally, *E. coli* is not known to metabolize mevalonate. Taken together, our mevalonate supplementation studies and the high production of PHB from acetyl-CoA suggested that the production of amorphadiene was limited by the conversion of acetyl-CoA to mevalonate.

3.2. Increased expression of the MevT operon inhibits cell growth

To increase the *in vivo* production of mevalonate we sought to increase the expression of the first three genes of the mevalonate pathway by increasing promoter strength and plasmid copy number. Specifically, the native *E. coli* *lac* promoter driving expression of MevT was changed from a non-consensus *lac* promoter to an arabinose-inducible *araBAD* promoter to create pBAD33MevT. Copy number was increased by using the medium copy plasmid pBAD24 (Guzman et al., 1995), also under arabinose control, to create pBAD24MevT.

Surprisingly increasing expression of the MevT genes resulted in the inhibition of cell growth. While induction of MevT, at 10–15 copies per cell, from the native P_{LAC} (pMevT) caused no significant change in cell growth, increased expression of the MevT operon from *araC*-P_{BAD} (pBAD33MevT) severely inhibited growth (Fig. 3). Increasing the copy number to 30–90 copies per cell (pBAD24MevT) resulted in even poorer growth. We hypothesized that the growth inhibition was due to either toxicity from the high expression of heterologous protein, leading to changes in cell metabolism collectively referred to as metabolic burden or load (Glick, 1995), or a specific alteration in cell metabolism caused by metabolites produced by the heterologous mevalonate pathway. As the data below demonstrate, increasing the expression of the MevT operon generated growth inhibition by the accumulation of specific mevalonate pathway metabolites.

3.3. HMGS activity is toxic while co-expression of tHMGR relieves growth inhibition

To determine the cause of growth inhibition associated with increased expression of the MevT operon, the operon was divided into its individual gene components and combinations thereof. The plasmids pAtoB, pHMGS, and pHMGR, each expressing one of the individual genes, and pBAD33 were transformed into *E. coli* DP10, and the resulting cells were incubated in a 96-well microtiter plate reader. Cell growth was monitored continuously after inducing with arabinose (Fig. 4A). In comparison to the empty plasmid control, expression of AACoAT and tHMGR alone had no effect on cell growth. However,

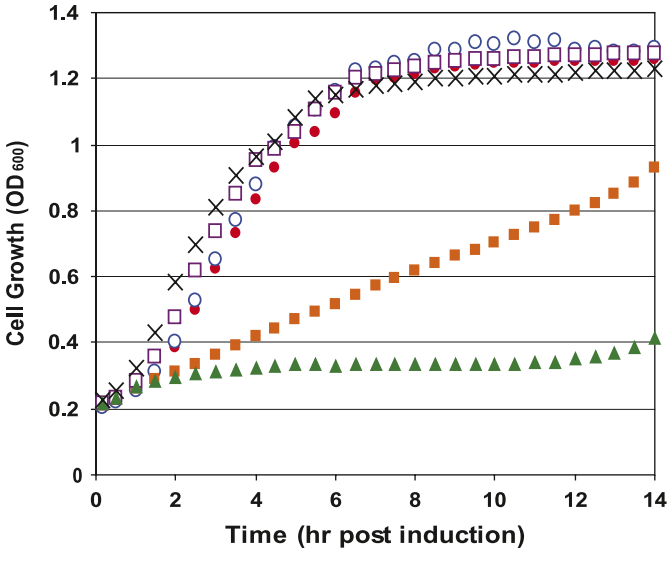


Fig. 3. Growth of *E. coli* with increasing expression of the MevT operon. Growth of *E. coli* DP10 harboring plasmids (in order of increasing expression of the MevT genes): pMevT (●), pBAD33MevT (■), pBAD24MevT (▲). pLac33 (○), pBAD33 (□), and pBAD24 (×) are the respective empty plasmid controls. Strains harboring pMevT and pLac33 were co-transformed with pTrc99A to control the unregulated P_{LAC} via $lacI^Q$ of pTrc99A.

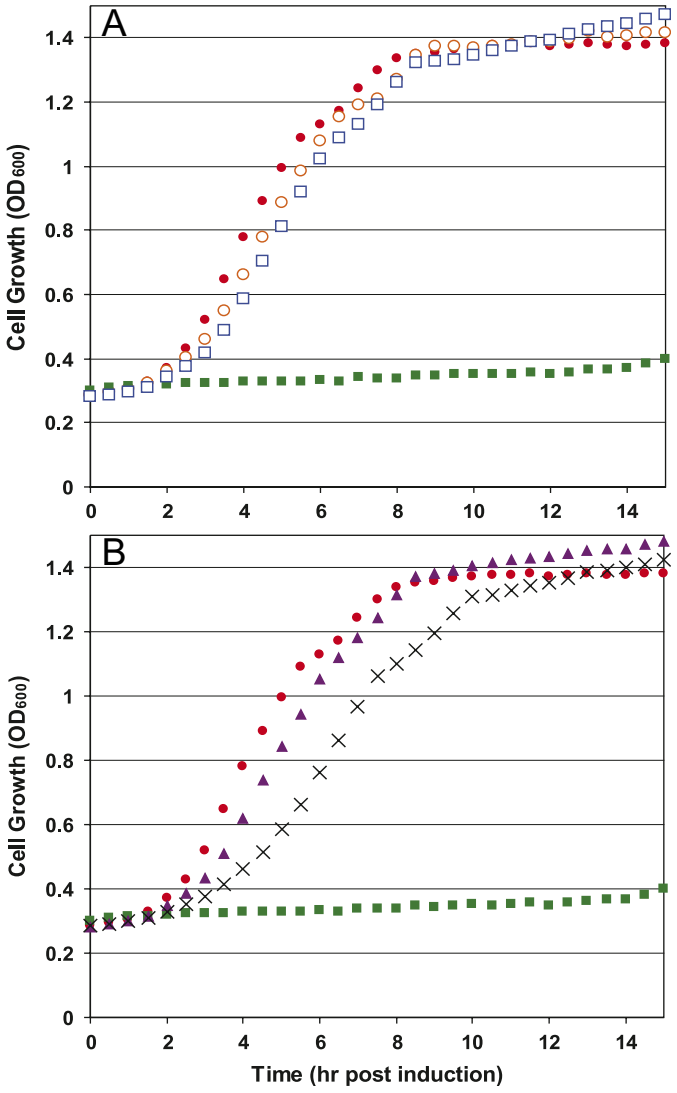


Fig. 4. Growth of *E. coli* DP10 expressing individual MevT genes and variants of pHMGS as described in Fig. 1. (A) Growth of cells harboring pBAD33 (empty plasmid control) (●), pAtoB (○), pHMGS (■), and pHMGR (□). (B) Growth of cells harboring empty plasmid control (●), pHMGS (■), pHMGS(C159A) containing inactive HMGS (▲), and pHMGSR (×).

expression of HMGS alone caused severe growth inhibition. This phenotype is linked to expression of HMGS, because under conditions that repress expression of HMGS from P_{BAD} (addition of 0.06% glucose to the media) pHMGS had little effect on growth (data not shown).

The growth inhibition caused by expression of HMGS may be due to the general burden caused by the high level production of a heterologous protein (Glick, 1995), to a metabolic load placed upon the cell by expressing a primary sequence containing rare amino acids (Bonomo and Gill, 2005), or the metabolic activity of HMGS. To differentiate between these possibilities, growth of cells expressing the active full-length HMGS was compared to cells expressing a catalytically inactive HMGS created by changing the catalytic cysteine to alanine (Rokosz et al., 1994) (Fig. 4B). As expected, the growth of cells expressing

active HMGS was inhibited, but strains harboring pHMGS(C159A) grew only slightly slower than the control cells harboring empty plasmids. From this we conclude that the heterologous protein production alone was not likely the cause of the observed growth defect.

E. coli is known to contain low levels of acetoacetyl-CoA (Pauli and Overath, 1972), which we confirmed by measuring *in vivo* acetoacetyl-CoA levels in wild-type *E. coli* (data not shown). To determine if activity of HMGS caused growth inhibition by the accumulation of the product, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), tHMGR was co-expressed with HMGS to convert, and thereby remove or detoxify, the HMG-CoA produced by HMGS. Introduction of tHMGR into cells expressing HMGS restored growth to that of the control (Fig. 4B). By co-expressing HMGS with tHMGR, *E. coli's* pool of acetoacetyl-CoA was still being consumed, as when HMGS was expressed alone, implying that the growth inhibition caused by HMGS expression was due to the accumulation of HMG-CoA and not the depletion of acetoacetyl-CoA. By expressing both genes, the HMG-CoA that is produced by HMGS is converted into mevalonate which then crosses the cell membrane into the medium with no observable growth inhibition (Fig. 4B). High extra-cellular concentrations of mevalonate had no significant effect on the growth of non-engineered *E. coli* (Martin et al., 2003).

To further eliminate acetoacetyl-CoA depletion as a possible cause of growth inhibition, strains harboring pHMGS, pHMGS(C159A) or pBAD33 were grown in the presence of increasing concentrations of acetoacetate, which is converted to acetoacetyl-CoA by the action of AtoA and AtoD (Pauli and Overath, 1972). No relief of toxicity was observed at any concentration, and the growth of all strains slowed with the addition of greater than 40 mM acetoacetate (data not shown), as reported previously (Potezny et al., 1981). These data further suggest that intracellular accumulation of HMG-CoA was the source of the toxicity because supplementing *E. coli* with acetoacetyl-CoA by the addition of extracellular acetoacetate did not alleviate the growth inhibition caused by HMGS activity.

3.4. The accumulation of HMG-CoA inhibits the growth of *E. coli*

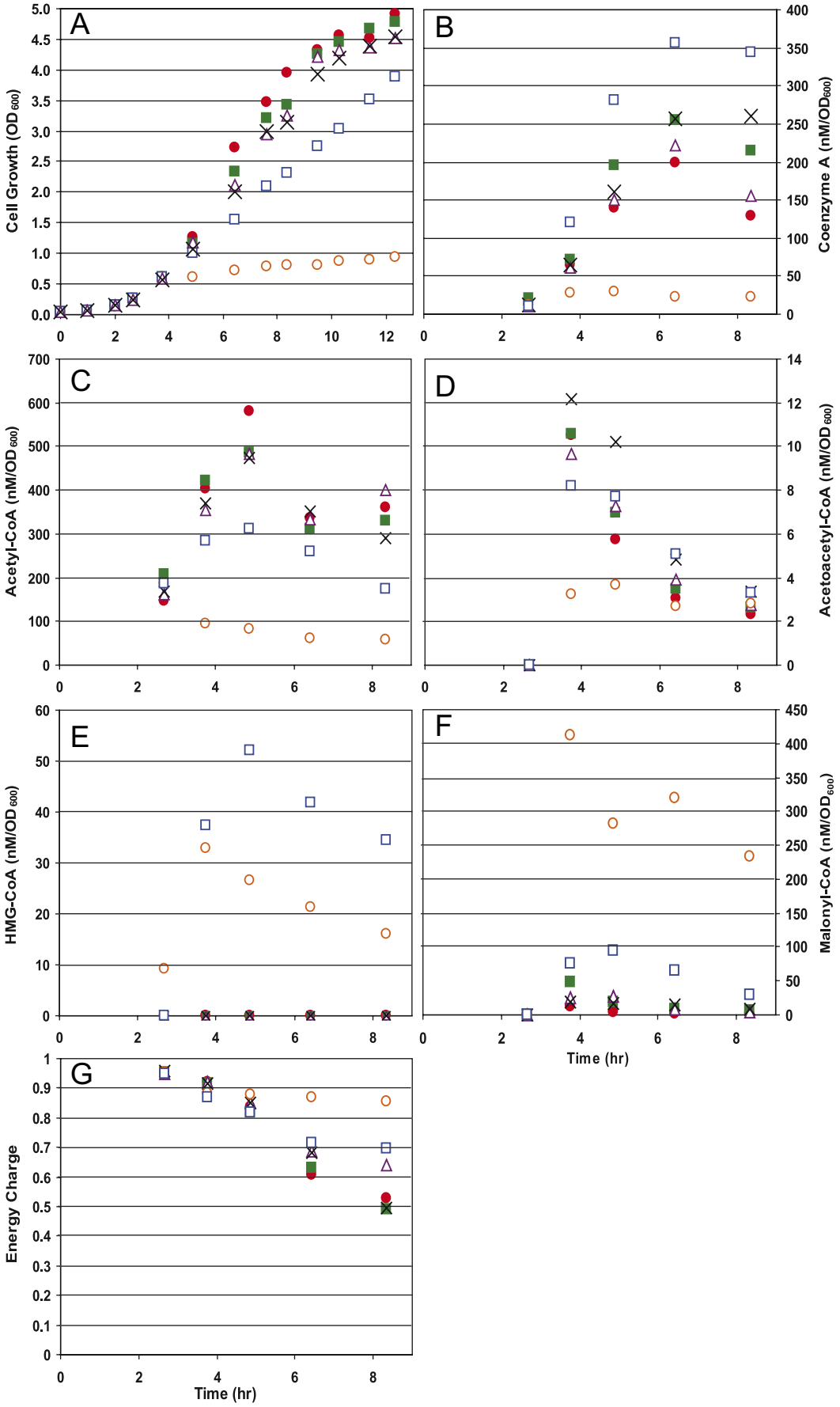
To elucidate the mechanism of growth inhibition in strains expressing the MevT operon and *HMGS* alone, the accumulation of intracellular metabolites was measured. Intracellular acyl-CoAs and adenylates were extracted from engineered strains at multiple time points beginning immediately prior to induction. Expression of the complete MevT operon from a strong promoter (pBAD33MevT) resulted in slowed cell growth and HMG-CoA accumulation (Fig. 5A and E). Growth of *E. coli* expressing HMGS alone was almost completely inhibited (Fig. 5A), which also correlated with an increase in HMG-CoA accumulation, even prior to induction (Fig. 5E). In comparison, cells co-expressing HMGS and tHMGR from the same transcript did not accumulate measurable levels of HMG-CoA (Fig. 5E) and grew to near wild-type densities. As expected, cells expressing HMGS(C159A) (alone or in the pMevT operon) also exhibited no growth inhibition (Fig. 5A) and did not accumulate measurable HMG-CoA (Fig. 5E). Taken together, these results indicate that the slow growth phenotype is linked to the accumulation of HMG-CoA.

Co-expression of HMGS and tHMGR appeared to efficiently convert the HMG-CoA produced to mevalonate (Table 2), thereby eliminating accumulation of the intermediate (Fig. 5E). In comparison, concurrent expression of AACoAT, HMGS, and tHMGR (pBAD33MevT) provided increased substrate for HMGS and greatly increased carbon flux through the initial mevalonate pathway, as shown by the approximately 125-fold increase in mevalonate produced (Table 2). However, with the additional carbon flux through the pathway, HMG-CoA began to accumulate after induction in cells harboring pBAD33-MevT (Fig. 5E), indicating unbalanced enzyme activity.

Examination of acetyl-CoA (Fig. 5C) levels revealed that cells harboring pHMGS and pBAD33MevT showed a moderate decrease in this metabolite. Cells expressing HMGS alone also exhibited lower acetoacetyl-CoA levels at early time points (Fig. 5D). However, if the depletion of acetyl-CoA and acetoacetyl-CoA by HMGS were the major cause of growth inhibition, we would expect a similar phenotype in strains co-expressing HMGS and tHMGR, which was not observed. It is feasible that including *tHMG1* in an operon with *HMGS* destabilized the transcript encoding both enzymes and reduced the expression of HMGS, leading to the above results. To eliminate this possibility, *HMGS* and *tHMG1* were co-expressed on separate plasmids (pHMGS and pBAD18HMGR) and similar results were obtained (data not shown).

Coenzyme A levels were also significantly reduced in strains harboring pHMGS in comparison to strains harboring pHMGSR, the empty vector control, or inactive pathway controls (Fig. 5B). Although coenzyme A depletion could account for growth inhibition, coenzyme A levels slightly increased upon expression of the full MevT operon in cells harboring pBAD33MevT.

Fig. 5. Analysis of strains expressing MevT genes. Cell growth, acyl-CoA levels and adenylate energy charge of *E. coli* DP10 harboring empty vector control (pBAD33) (●), or expressing the following protein or proteins: HMGS (pHMGS) (○), HMGS + tHMGR (pHMGSR) (■), AACoAT + HMGS + tHMGR (pBad33MevT) (□), inactive HMGS (pHMGS(C159A)) (△), and AACoAT + inactive HMGS + tHMGR (pMevT (C159A); inactive MevT) (×). (A) Cell growth, (B) Coenzyme A, (C) acetyl-CoA, (D) acetoacetyl-CoA, (E) HMG-CoA, (F) malonyl-CoA, and (G) adenylate energy charge. The first sample for metabolite extraction was taken immediately prior to induction. The experiment was repeated in duplicate and identical trends were obtained.


A
B
C
D
E
F
G
Cell Growth (OD600)
Coenzyme A (nM/OD600)
Acetyl-CoA (nM/OD600)
Acetoacetyl-CoA (nM/OD600)
HMG-CoA (nM/OD600)
Malonyl-CoA (nM/OD600)
Energy Charge
Time (hr)
Time (hr)

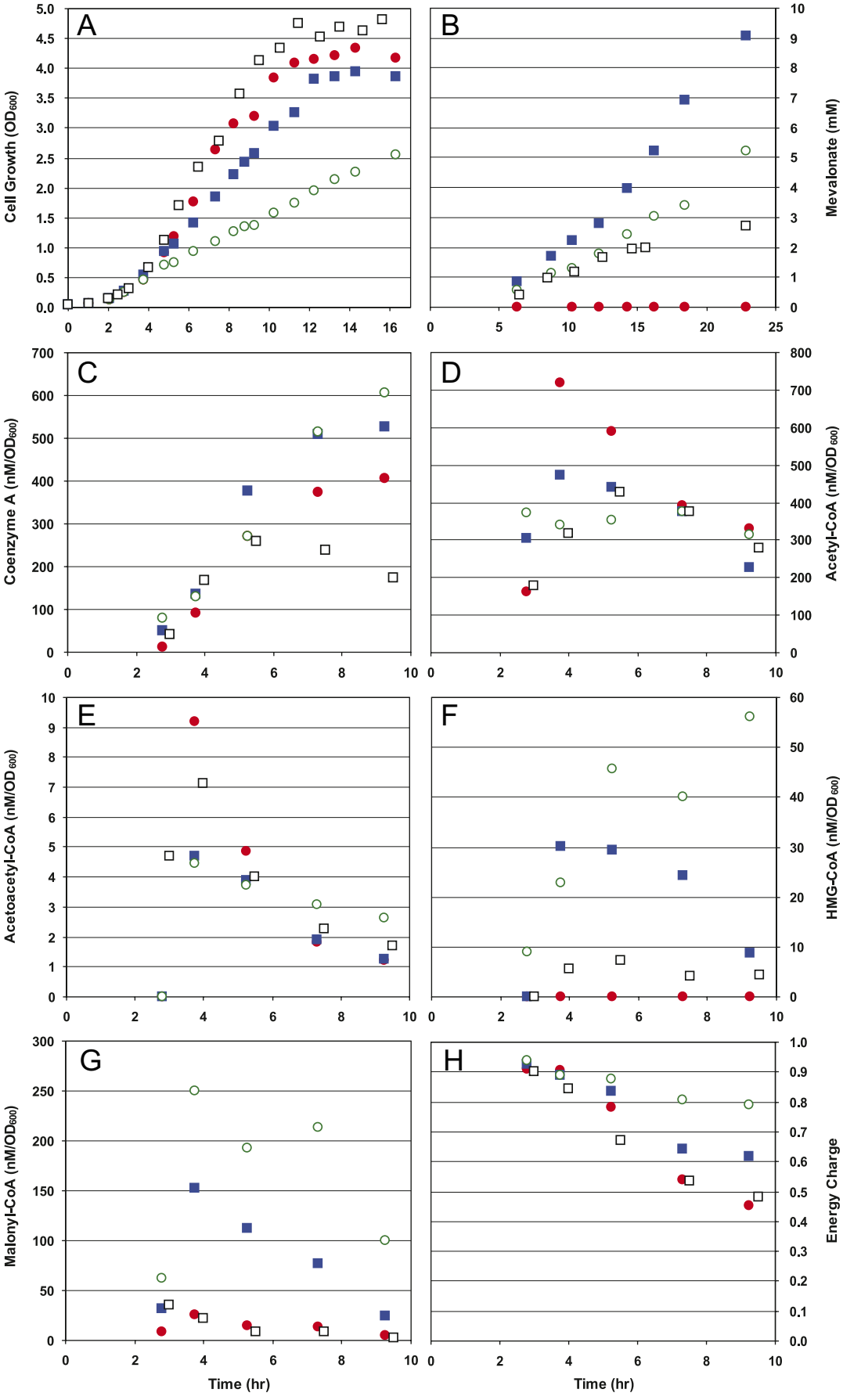

A
B
C
D
E
F
G
H
Cell Growth (OD600)
Mevalonate (mM)
Coenzyme A (nM/OD600)
Acetyl-CoA (nM/OD600)
Acetoacetyl-CoA (nM/OD600)
HMG-CoA (nM/OD600)
Malonyl-CoA (nM/OD600)
Energy Charge
Time (hr)
Time (hr)

Table 2
Mevalonate production from *E. coli* DP10 expressing MevT genes (see Fig. 5)

	Mevalonate concentration at 25 h (mM)
pHMGSR	0.12
pBAD33MevT	15.21
pMevT(C159A)	n.d.

Interestingly, growth-inhibited strains that accumulated HMG-CoA simultaneously accumulated the fatty acid precursor malonyl-CoA (Fig. 5F) in proportion to initial HMG-CoA levels. The high concentrations of malonyl-CoA may account for the low levels of coenzyme A, acetyl-CoA, and acetoacetyl-CoA when expressing HMGS alone, by effectively trapping free coenzyme A.

The adenylate energy charge (EC) of all cultures were >0.8 during early logarithmic growth (Fig. 5G), indicating rapid quenching of cellular metabolism and negligible degradation of adenylates or acyl-CoAs during extraction from the cell (Chapman et al., 1971). However, cells harboring pHMGS and pBAD33MevT had higher energy charges at later times than strains that did not experience growth inhibition (Fig. 5G). Energy charge has not been shown to change substantially with the production of heterologous protein (Hoffmann et al., 2002), therefore the accumulation of HMG-CoA appears to be altering cellular metabolism.

The remaining acyl-CoAs that were tracked (propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA) did not vary significantly among strains.

3.5. Increasing expression of tHMGR reduces toxicity caused by overexpression of MevT

To restore the growth of cells inhibited by HMG-CoA accumulation, an additional copy of *tHMG1* was introduced on a second, medium-copy plasmid (pBAD33MevT and pBAD18HMGR). Cells expressing extra copies of *tHMG1* increased the growth rate to almost that of the inactive control (Fig. 6A), reduced the accumulation of intracellular HMG-CoA (Fig. 6F), particularly at later times, and increased mevalonate production by almost two-fold over cells without a additional copies of *tHMG1* (Fig. 6B). The free coenzyme-A (Fig. 6C) and acetoacetyl-CoA (Fig. 6E) levels of both strains harboring pBAD33-MevT were nearly identical, and acetyl-CoA levels were similar (Fig. 6D). All strains with an active MevT operon had lower levels of acetyl-CoA at early time points, presumably from the conversion of *E. coli*'s pool of acetyl-CoA to mevalonate. Interestingly, cells harboring pBAD33MevT and pBAD18HMGR also accumulated less malonyl-CoA than cells with pBAD33MevT only, although the concentrations were higher than in the control strain (Fig. 6G).

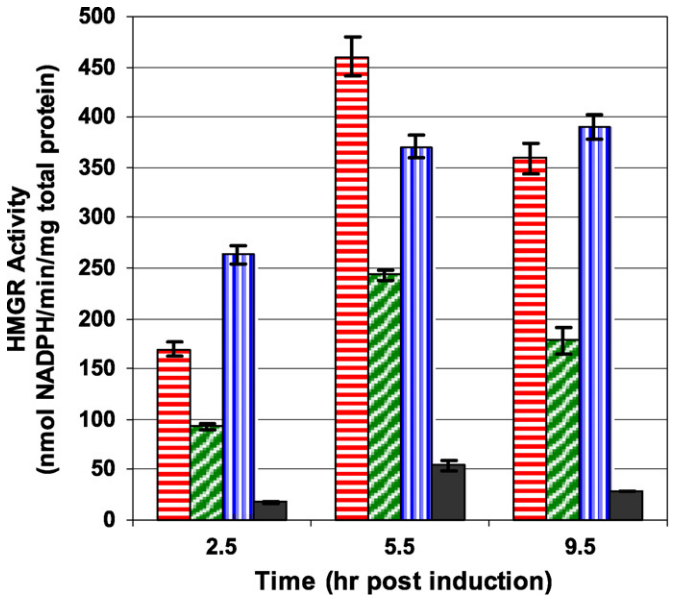


Fig. 7. HMG-CoA reductase activity of *E. coli* DP10 harboring two plasmid systems for the production of mevalonate, expressing the following proteins: AACoAT + inactive HMGS + tHMGR (pMevT (C159A) and pBAD18; inactive pathway control) (horizontal lines), AACoAT + HMGS + tHMGR (pBAD33MevT and pBAD18) (diagonal lines), AACoAT + HMGS + overexpressed tHMGR (pBAD33-MevT and pBAD18HMGR) (vertical lines), and AACoAT + HMGS + tHMGR expressed from a non-consensus Lac promoter (pMevT and pTrc99A) (solid bars). Cell cultures were induced at OD_{600} of approximately 0.25. The assay detected no HMG-CoA reductase activity from lysates of empty vector control cells harboring pBAD33 and pBAD18.

The increased copy number of *tHMG1* resulted in a peak increase in cellular HMG-CoA reductase activity 2.5 fold above cells harboring pBAD33MevT alone (Fig. 7). In comparison, expression of MevT alone from the non-consensus P_{LAC} (pMevT) resulted in substantially less HMGR activity than when the operon was expressed from *araC*-P_{BAD} (pBAD33MevT). Reduced expression of MevT resulted in less accumulation of HMG-CoA (Fig. 6F) from the unbalanced operon and more rapid cell growth (Fig. 6A); however, reduced expression also resulted in the production of less mevalonate (Fig. 6B). Interestingly,

Fig. 6. Analysis of cells over-expressing *tHMG1* and MevT operons. Cell growth, acyl-CoA levels and adenylate energy charge of *E. coli* DP10 harboring two plasmid systems for the production of mevalonate expressing the following proteins: AACoAT + inactive HMGS + tHMGR (pMevT(C159A) and pBAD18; inactive pathway control) (●), AACoAT + HMGS + tHMGR (pBAD33MevT and pBAD18) (○),AACoAT + HMGS + overexpressed tHMGR (pBAD33MevT and pBAD18HMGR) (■), AACoAT + HMGS + tHMGR expressed from a non-consensus Lac promoter (pMevT and pTrc99A) (□). (A) Cell growth, (B) mevalonate, (C) coenzyme A, (D) acetyl-CoA, (E) acetoacetyl-CoA, (F) HMG-CoA, (G) malonyl-CoA, and (H) adenylate energy charge. The first sample for metabolite extraction was taken immediately prior to induction except for pMevT and pTrc99A which was taken 0.5 h post induction. The experiment was repeated in duplicate and identical trends were obtained.

HMGR activity of the strain with the inactive HMGS was substantially higher than that of the strain with the active MevT operon in pBAD33MevT. Although expression of this construct produced active tHMGR, there was no metabolic flux through the pathway and also no measured growth inhibition.

All four strains contained relatively similar levels of acetoacetyl-CoA (Fig. 6E) that decreased over time, regardless of growth rate, verifying that the depletion of acetoacetyl-CoA was not the cause of growth inhibition. Distinct differences were seen in the levels of coenzyme A (Fig. 6C) at later times; however, the coenzyme A pool was lowest in strains that displayed little or no growth inhibition.

From these data, it is clear that growth inhibition in strains overexpressing the MevT operon was caused by the accumulation of intracellular HMG-CoA and not the metabolic burden of heterologous protein production or the depletion of acyl-CoA precursors. By co-expressing the MevT operon and extra copies of *tHMG1* from *araC*-P_{BAD}, growth inhibition and HMG-CoA accumulation was reduced leading to a three-fold increase in mevalonate production over our initial mevalonate producing plasmid, pMevT. Implementing a similar optimization strategy with the remaining mevalonate pathway genes and amorphadiene synthase should result in increased production of the sesquiterpene. In addition, increasing the total activity of tHMGR and alleviating the toxicity caused by the high expression of MevT would increase the production of any isoprenoid whose downstream biosynthetic genes were also properly expressed.

4. Discussion

Examination of metabolites of a mevalonate pathway expressed in *E. coli* revealed that this heterologous pathway is unbalanced, resulting in growth inhibition in some engineered strains with high expression levels of the MevT operon. Specifically, we show that HMG-CoA accumulates in cells with high MevT expression and this metabolite inhibits cell growth. Co-expression with additional tHMGR, which converts HMG-CoA to non-toxic mevalonate, relieves this toxicity and restores growth nearly to the levels seen in control strains.

The HMG-CoA produced is not native to the *E. coli* cytosol, it is not known to be acted upon by any enzyme in *E. coli,* and, as in the case of other acyl-CoAs, it is not expected to cross the cell membrane. Therefore, HMG-CoA accumulates in the cell, as seen by metabolite analysis of the affected strains. We hypothesize that once the HMG-CoA concentration reached a threshold level it inhibited cellular processes and thus inhibited cell growth. Cells harboring pHMGS began accumulating HMG-CoA even prior to induction. Once the HMG-CoA concentration reached the threshold level, intracellular processes and cell growth were inhibited. With co-expression of both HMGS and tHMGR, HMG-CoA produced was converted to mevalonate, as evidenced by the lack of even transient levels of HMG-CoA in cells harboring pHMGSR.

The equilibrium of the acetyl transferase reaction, catalyzed by AACoAT, is heavily weighted toward the formation of acetyl-CoA in *E. coli* as in yeast (Middleton, 1972; Duncombe and Frerman, 1976). However, the ability of the thiolase to quickly replace acetoacetyl-CoA consumed by HMGS leads to high flux to HMG-CoA and, with the expression of HMGR, high flux to mevalonate. Despite the high flux to mevalonate, the activity of HMGS appears to overcome the activity of HMGR, resulting in an unbalanced pathway and an accumulation of HMG-CoA in the case of pBAD33MevT. Thus, despite the presence of an outlet for HMG-CoA, provided by tHMGR, high expression of AACoAT with HMGS resulted in a growth inhibition similar to that seen in the expression of *HMGS* alone.

Increased expression of AACoAT provided increased substrate for HMGS and substantially increased the production of mevalonate, as was shown by the 125-fold difference in production between cells harboring pBad33-MevT or pHMGSR. A modest 13% increase in mevalonate production was found when co-expressing a heterologous acetyl transferase (thiolase), HMG-CoA synthase, and HMG-CoA reductase from a *Streptomyces* sp. strain CL190 in *S. lividans* (Kuzuyama et al., 2004). This limited increase in mevalonate production may be due to a pathway imbalance created by the use of a heterologous mevalonate pathway.

We have noted that HMG-CoA accumulates to a higher level in cells containing pBAD33MevT than cells harboring pHMGS, a strain that exhibits a more severe growth defect. However, HMG-CoA accumulates more rapidly and earlier in cell growth in cells expressing HMGS alone. Several lines of evidence indicate that the rapid accumulation of HMG-CoA in cells expressing HMGS alone leads to a global slowing of cellular metabolism that would be expected from the growth defect. From surveying the various CoA derivatives, it is clear that cells expressing HMGS alone contain much less of all the measured CoAs, except malonyl-CoA, which may be the cause of the severe growth inhibition. Further, it has been shown that the cessation of anabolism, after the inhibition of cellular translation or transcription (by the addition of chloramphenicol or rifampicin, respectively) results in a sustained, constant energy charge (Andersen and von Meyenburg, 1977), similar to that observed for cells harboring pHMGS. Thus, it may be that after the HMG-CoA concentration reaches an inhibitory level the cells are static and anabolic reactions are likely reduced. In comparison, cells harboring pBAD33MevT accumulated HMG-CoA later in cell growth causing a reduction in growth, but not complete cessation. As the strain continues to slowly grow, it builds up a higher level of HMG-CoA.

The toxicity caused by the accumulation of HMG-CoA has not been reported in bacteria that employ the DXP isoprenoid pathway, such as *E. coli*, but may have been

observed in bacteria that naturally contain a mevalonate isoprenoid pathway. Wilding et al. (2000) noted that in studying the bacterium *Streptococcus pneumoniae*, they could obtain mutants grown in the presence of mevalonate that were either null for the gene corresponding to HMG-CoA synthase or null for the genes corresponding to both HMG-CoA synthase and HMG-CoA reductase. However, they could not obtain a mutant in only the gene corresponding to HMG-CoA reductase. It is possible that a null mutation in *S. pneumoniae* HMG-CoA reductase could lead to a lethal intracellular accumulation of HMG-CoA; however, this was not proven.

The problem of HMG-CoA toxicity in *E. coli* may have been observed but not recognized in the past. Hamano et al. (2001) reported that attempts to place a construct containing a mevalonate pathway operon from *Streptomyces griseolosporeus* MF730-N6 into *E. coli* were unsuccessful on a high-copy plasmid, whereas a low-copy plasmid allowed *E. coli* to be transformed with this construct. Despite successful uptake of the foreign DNA, cells harboring the low-copy plasmid still grew poorly in liquid media.

In its native host, yeast HMGR transcription and stability is well regulated, and HMG-CoA may not accumulate to any appreciable amount (Hampton et al., 1996; Hampton and Bhakta, 1997). Alternatively, the target of intracellular HMG-CoA in *E. coli* may not exist in yeast, the eukaryotic cell may be resistant to interference from this molecule, or HMG-CoA may be sequestered in a different organelle from its targets. Higher eukaryotes can also convert HMG-CoA into acetyl-CoA and acetoacetyl-CoA through the action of HMG-CoA lyase, a key step of both ketogenesis and leucine catabolism (Ashmarina et al., 1999).

How HMG-CoA causes growth inhibition in *E. coli* has not been elucidated in this study. Intracellular coenzyme A is often limiting in rapidly growing *E. coli,* and a deficiency leads to an arrest in protein synthesis and cell growth (Jackowski and Rock, 1986). Sequestering the supply of free coenzyme A in the cell in the form of HMG-CoA or malonyl-CoA could cause a deficiency and lead to growth inhibition. However, we only observed a depletion of free coenzyme A when HMGS was expressed alone. The overexpression of the full MevT operon, while still causing growth inhibition, actually resulted in slightly increased free coenzyme A levels in comparison to the control strains. Although coenzyme A depletion may have exacerbated the problem, it does not appear to be the underlying cause of the observed phenotype.

We also observed that malonyl-CoA simultaneously accumulates with HMG-CoA in cells that are growth inhibited. Malonyl-CoA is only utilized in *E. coli* as a precursor for fatty acid biosynthesis through the type II (disassociated) fatty acid synthesis pathway (Rock and Cronan, 1996; Subrahmanyam and Cronan, 1998). Reports have shown that blocking the early steps of the fatty acid biosynthetic pathway in *E. coli* results in growth inhibition from fatty acid limitation and causes the accumulation of malonyl-CoA at the expense of acetyl-CoA (Furukawa et al., 1993; Heath and Rock, 1995). Furthermore, antibiotics that mimic the acetylester moiety of malonyl-CoA have been shown to selectively inhibit the type II fatty acid biosynthesis pathway and not the type I pathway found in non-plant eukaryotes, such as *S. cerevisiae* (Furukawa et al., 1993), again leading to growth inhibition of cells relying on type II fatty acid biosynthesis coincident with the accumulation of malonyl-CoA. There is no known enzyme that converts HMG-CoA to malonyl-CoA, but it is possible that high intracellular levels of HMG-CoA inhibit an enzyme or enzymes in the early steps of *E. coli*'s type II fatty acid biosynthetic pathway, by a method similar to the antibiotics selective for the type II pathway discussed above. Such enzyme inhibition would likely lead to reduction or cessation of cell growth and accumulation of malonyl-CoA. However, further study is required.

Regardless of the biochemical effect of HMG-CoA accumulation, the accumulation implies that there exists an imbalance between the production of HMG-CoA and its subsequent conversion to mevalonate in the engineered mevalonate pathway. By increasing production of tHMGR and thereby increasing the total activity of the enzyme, HMG-CoA levels were reduced and the growth inhibition was overcome, resulting in increased production of mevalonate. This solution was obtained, in part, by profiling the heterologous pathway metabolites in actively growing and growth-inhibited cells to identify toxic intermediates. Alternatively, combinatorially changing the expression of each gene in the operon, relative to each other, would likely result in a similar solution. The activities of the pathway enzymes may also be balanced by inserting a library of various strength promoters 5′ to each gene (Alper et al., 2005a), altering the strength of ribosome binding sites (Barrick et al., 1994), tuning post-transcriptional control elements (Pfleger et al., 2006b), or through directed evolution (Lutz and Patrick, 2004). However, a relatively high-throughput screen for the desired product would be required to assess improvements from the library of varied pathway enzyme activity combinations (Pfleger et al., 2006a).

Our results demonstrate that balancing flux through the heterologous pathway is a key determinant in optimizing isoprenoid production in microbial hosts. The results of this study present a model system for engineering microbes for the production of bioactive molecules of therapeutic and industrial value. Current research in metabolic engineering and the new focus of synthetic biology seek to create novel biological functions by rebuilding cellular metabolism through the modification and integration of well characterized genetic components (Voigt and Keasling, 2005). However, in the combinatorial construction of large pathways from components from different sources, the resulting carbon flux frequently becomes imbalanced, leading to the accumulation of intermediates that lower the efficiency of the system, at best, or are detrimental to the engineered host for often unforeseen reasons.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2006.11.002.

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