nature biotechnology

Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids

Vincent JJ Martin[1,2,3], Douglas J Pitera[1,3], Sydnor T Withers[1], Jack D Newman[1] & Jay D Keasling[1]

Isoprenoids are the most numerous and structurally diverse family of natural products. Terpenoids, a class of isoprenoids often isolated from plants, are used as commercial flavor and fragrance compounds and antimalarial or anticancer drugs. Because plant tissue extractions typically yield low terpenoid concentrations, we sought an alternative method to produce high-value terpenoid compounds, such as the antimalarial drug artemisinin, in a microbial host. We engineered the expression of a synthetic amorpha-4,11-diene synthase gene and the mevalonate isoprenoid pathway from *Saccharomyces cerevisiae* in *Escherichia coli*. Concentrations of amorphadiene, the sesquiterpene olefin precursor to artemisinin, reached 24 μg caryophyllene equivalent/ml. Because isopentenyl and dimethylallyl pyrophosphates are the universal precursors to all isoprenoids, the strains developed in this study can serve as platform hosts for the production of any terpenoid compound for which a terpene synthase gene is available.

Terpenoids comprise a highly diverse class of natural products from which numerous commercial flavors, fragrances and medicines are derived. These valuable compounds are commonly isolated from plants, microbes and marine organisms. For example, terpenoids extracted from plants are used as anticancer and antimalarial drugs[1,2]. Because these compounds are naturally produced in small quantities, purification from biological material suffers from low yields, impurities and consumption of large amounts of natural resources. Furthermore, because of the complexity of these molecules, the chemical syntheses of terpenoids are inherently difficult and expensive and produce relatively low yields[3–5]. For these reasons, the engineering of metabolic pathways to produce large quantities of complex terpenoids in a tractable biological host presents an attractive alternative to extractions from environmental sources or chemical syntheses.

Here we describe the production of amorpha-4,11-diene from the bacterium *E. coli*. Amorphadiene is the sesquiterpene olefin precursor to artemisinin, a valuable and powerful antimalarial natural product first isolated from sweet wormwood or *Artemisia annua*. In certain regions of the world, strains of *Plasmodium* have emerged that are resistant to the traditional antimalarial drugs of choice, such as chloroquine, mefloquine, halofantrinc, quinine and the sulfadoxine-pyrimethamine combination. Artemisinins have been acclaimed as the next generation of antimalarial drugs because they show little or no cross-resistance with existing antimalarials[6–8]. Commercial production of artemisinin currently relies on its extraction and purification from plant material and, as would be expected, the yields are low[9]. Artemisinin is but one example of a group of terpene-based natural products that have been used in treating human disease. These include Taxol, a diterpene extracted from the Pacific yew that is extremely effective in the treatment of certain cancers[10,11], and irufloven, a third-generation semisynthetic analog of the sesquiterpene illudin S that are in late-stage clinical trials for the treatment of various refractory and relapsed cancers[12,13]. In general, these drugs are extracted from the host plant, in which they accumulate in very small amounts, before further derivatization or use.

To eliminate the need for plant extraction, we sought to produce terpenoid compounds at high yields in a microbial host by introducing a heterologous, high-flux isoprenoid pathway into *E. coli*. Although most terpene olefins are active when derivatized, the ability to produce the olefin backbone in large quantities in a genetically and metabolically tractable host represents an important step toward producing terpenoid-based drugs in large-scale fermentations. Because all terpenoids are produced from the same universal precursors, host microbes engineered to produce copious quantities of these precursors may be used to biosynthesize any terpene.

Two isoprenoid biosynthetic pathways exist that synthesize the precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (**Fig. 1**). Eukaryotes other than plants use the mevalonate-dependent (MEV) isoprenoid pathway exclusively to convert acetyl-coenzyme A (acetyl-CoA) to IPP, which is subsequently isomerized to DMAPP. Plants use both the MEV and the mevalonate-independent, or deoxyxylulose 5-phosphate (DXP), pathways for isoprenoid synthesis. Prokaryotes, with some exceptions[14], use the DXP pathway to produce IPP and DMAPP separately through a branch point[15] (**Fig. 1**). IPP and DMAPP precursors are essential to *E. coli* for the prenylation of tRNAs[16] and the synthesis of farnesyl pyrophosphate (FPP), which is used for quinone and cell wall biosynthesis.

Several groups have described the engineering of the DXP pathway to increase the supply of isoprenoid precursors needed for

[1]Department of Chemical Engineering, 201 Gilman Hall, University of California, Berkeley, California 94720-1462, USA. [2]Lawrence Berkeley National Laboratory, 1 Cyclotron Road (HILD 201), Berkeley, California 94720, USA. [3]These authors contributed equally to this work. Correspondence should be addressed to J.D.K. (keasling@socrates.berkeley.edu).

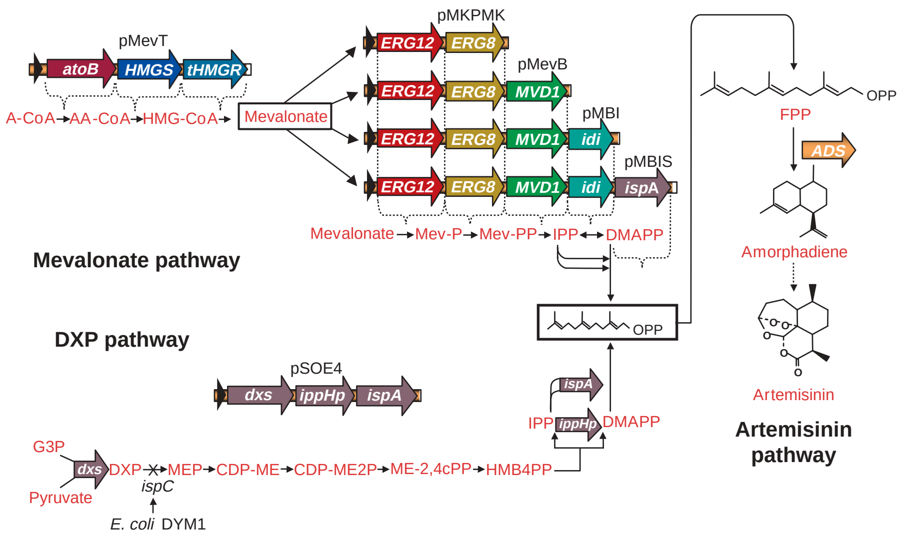

Figure 1 Production of amorphadiene via the DXP or mevalonate isoprenoid pathways and depiction of the synthetic operons used in this study. Black triangles represent the P_{LAC} promoter and tHMGR refers to an N-terminal truncated product of the native *HMGR* gene. Gene symbols and the enzymes they encode (all genes were isolated from *S. cerevisiae* except where noted): *atoB*, acetoacetyl-CoA thiolase from *E. coli*; *HMGS*, HMG-CoA synthase; *tHMGR*, truncated HMG-CoA reductase; *ERG1*, mevalonate kinase; *ERG8*, phosphomevalonate kinase; *MVD1*, mevalonate pyrophosphate decarboxylase; *idi*, IPP isomerase from *E. coli*; *ippHp*, IPP isomerase from *Haematococcus pluvialis*; *dxs*, 1-deoxy-D-xylulose 5-phosphate synthase; *ispC*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *ispA*, FPP synthase from *E. coli*; *ADS*, amorphadiene synthase. Pathway intermediates: G3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; ME-2,4cPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; HMB4PP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; A-CoA, acetyl-CoA; AA-CoA acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; Mev-P, mevalonate 5-phosphate; Mev-PP, mevalonate pyrophosphate.

high-level production of carotenoids in *E. coli*[17–19]. Balancing the pool of glyceraldehyde-3-phosphate and pyruvate, or increasing the expression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS; encoded by the gene *dxs*) and IPP isomerase (encoded by *idi*), resulted in increased carotenoid buildup in the cell. Though improvements in isoprenoid production were noted, this approach most likely suffered from limitations owing to control mechanisms present in the native host. Because the DXP pathway may be tied to unknown physiological control elements in *E. coli*, we chose to bypass this pathway by engineering the expression of the *S. cerevisiae* mevalonate-dependent pathway in *E. coli*. We found that expression of this heterologous pathway in *E. coli* led to such an abundance of isoprenoid precursors that cells either ceased to grow or mutated to overcome the toxicity. The simultaneous expression of a synthetic amorphadiene synthase gene[20] in our engineered strain resulted in high-level production of amorphadiene and alleviated growth inhibition. Because IPP and DMAPP are the universal precursors to all isoprenoids, the strains reported here can serve as platform hosts for the production of any terpenoid compound for which the biosynthetic genes are available.

RESULTS

Synthase gene assembly and amorphadiene production

Previous studies on the production of sesquiterpenes using native plant genes established that poor expression of the plant genes in *E. coli* restricted the terpene yields[21]. To overcome the difficulties in expressing terpene synthases and to achieve high-level production of the artemisinin precursor amorphadiene, we synthesized and expressed a codon-optimized variant of *ADS*, the gene encoding amorphadiene synthase, designed for high-level expression in *E. coli*. By expressing a codon-optimized synthase, we hoped to shift the limitation of microbial terpene synthesis from expression of the synthase gene to supply of the precursor (FPP) by the isoprenoid pathway. The *ADS* gene synthesis, which used a two-step assembly and a one-step amplification PCR, yielded the expected 1.7 kb product. Sequence analysis of three *ADS* genes from independent clones identified two mutations or more in each of the genes. A functional *ADS* gene was assembled from two clones and by means of two site-directed mutagenesis reactions. Expression of the synthetic *ADS* gene in *E. coli* DH10B resulted in a peak concentration of amorphadiene of 0.086 µg caryophyllene equivalent/ml/OD_{600} after 10 h of growth in LB medium (**Fig. 2a**). The peak concentration of amorphadiene increased to 0.313 µg caryophyllene equivalent/ml/OD_{600} (**Fig. 2a**) upon coexpression with the SOE4 operon encoding DXS, IPPHp and IspA (**Fig. 1**), which are rate-limiting enzymes of the native DXP isoprenoid pathway. Given this 3.6-fold increase in amorphadiene concentration upon coexpression, we suspected that FPP synthesis and not *ADS* expression limited amorphadiene production in this engineered host.

Engineering the mevalonate-dependent pathway in *E. coli*

To increase the intracellular concentration of FPP substrate supplied to the amorphadiene synthase, we assembled the genes encoding the mevalonate-dependent isoprenoid pathway from *S. cerevisiae* into operons and expressed them in *E. coli*. To simplify the task of engineering an eight-gene biosynthetic pathway, we divided the genes into two operons, referred to as 'top' and 'bottom.' The 'top' operon, MevT, transforms the ubiquitous precursor acetyl-CoA to *(R)*-mevalonate in three enzymatic steps (**Fig. 1**). The 'bottom' operon converts the *(R)*-mevalonate to IPP, DMAPP and/or FPP depending on the construct (**Fig. 1**). To test the functionality of the heterologous pathway, an *E. coli* strain deficient in isoprenoid synthesis (strain DYM1) was transformed with plasmids expressing the three different bottom operon constructs pMevB, pMBI and pMBIS (**Fig. 1**). Strain DYM1 has a deletion in the *ispC* gene[22] and therefore cannot synthesize 2-*C*-methyl-D-erythritol 4-phosphate, an intermediate in the endogenous isoprenoid biosynthetic pathway (**Fig. 1**). As expected, all strains grew in the presence of 2-*C*-methyl-D-erythritol, but only the strains harboring pMBI or pMBIS, and not pMevB, grew on plates supplemented with 1 mM mevalonate in the absence of methylerythritol (data not shown). These results established that the synthetic MBI and MBIS operons were functional and capable of supplying IPP and DMAPP required for the growth of *E. coli*. Because the DXP pathway supplies the cells with IPP and DMAPP from a branch point[15], a mutation in *ispC* prohibits the synthesis of both precursors. Although *E. coli* maintains a nonessential copy of the IPP isomerase gene on its chromosome[23], the

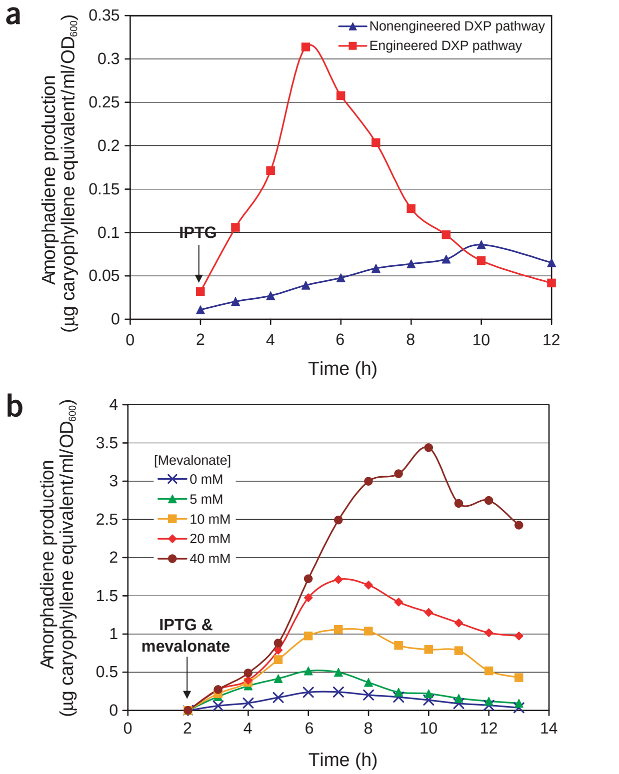

Figure 2 Comparison of the production of amorphadiene in LB medium. (**a**) Amorphadiene production by the synthetic amorphadiene synthase was measured from *E. coli* DH10B (nonengineered DXP pathway) and *E. coli* DH10B harboring the pSOE4 plasmid (engineered DXP pathway, **Fig. 1**) and (**b**) from *E. coli* DH10B expressing the mevalonate bottom operon (pMBIS, **Fig. 1**) in cultures supplemented with increasing amounts of DL-mevalonate. Because amorphadiene was not available commercially, its concentrations were reported as equivalents of caryophyllene, another sesquiterpene olefin, using a standard curve and the relative abundance of ions 189 and 204 *m/z* of the two compounds.

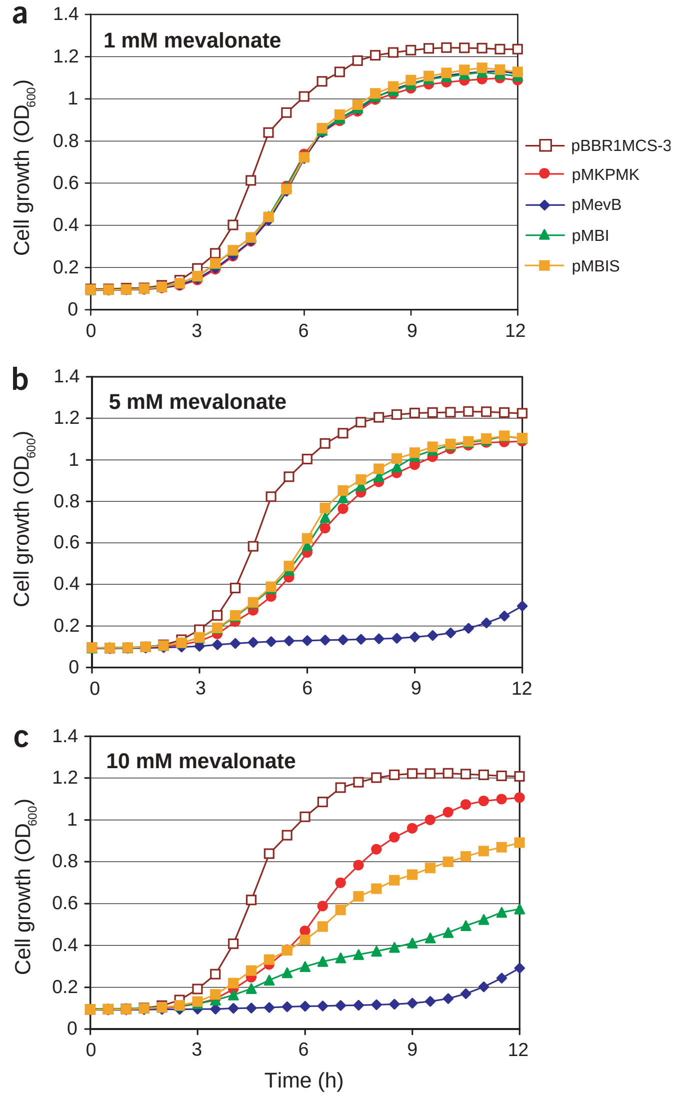

Figure 3 Growth curves of *E. coli* showing the inhibition effect caused by increasing concentrations of DL-mevalonate in the LB medium. The *E. coli* strains harbor either the pBBR1MCS-3 (empty plasmid control), pMKPMK, pMevB, pMBI or pMBIS plasmids expressing the various mevalonate operons described in **Figure 1**.

gene's expression seems to be too low to support the growth of *E. coli* when only IPP is supplied by the MevB operon.

To complete the mevalonate pathway and allow the synthesis of sesquiterpene precursors from a simple and inexpensive carbon source, the pMevT plasmid expressing the remaining three genes (*atoB*, *HMGS* and t*HMGR*) of the mevalonate isoprenoid pathway was transformed with either pMBI or pMBIS. Coexpression of the two operons, which together encode a complete pathway for the synthesis of isoprenoids from acetyl-CoA, complemented the *ispC* deletion even in the absence of mevalonate, indicating that the MevT operon was functional (data not shown).

Amorphadiene synthesis from mevalonate

To achieve high-level production of amorphadiene and to determine if the supply of FPP to the terpene synthase was limiting amorphadiene yields, the mevalonate pathway was coupled to amorphadiene synthesis in *E. coli*. Cells harboring the *ADS* gene coexpressed with the MBIS operon were grown in medium supplemented with exogenous mevalonate. Gas chromatography–mass spectrometry (GC-MS) analysis of the culture extracts showed that the peak amorphadiene concentration from these cultures was proportional to the amount of mevalonate added to the medium, up to a concentration of 40 mM mevalonate (**Fig. 2b**). These results indicated that flux from the MBIS operon did not limit amorphadiene production at the highest mevalonate concentration used. Cultures supplemented with 40 mM mevalonate produced a peak concentration of 3.4 µg caryophyllene equivalents/ml/OD_{600}, which is a 40- and 11-fold increase over the endogenous and engineered DXP pathway, respectively. The drop in amorphadiene concentration with time was due to the loss of the volatile terpene to the headspace, which means that these reported production values are certainly underestimated.

We observed severe growth inhibition upon addition of more than 10 mM mevalonate in the control cultures where the amorphadiene synthase was not expressed (**Fig. 3**). To investigate the cause of this inhibition, we measured the growth of *E. coli* DH10B from strains harboring either the pMKPMK, pMevB, pMBI or pMBIS plasmid in media supplemented with increasing concentrations of exogenous mevalonate. Although the addition of 5 mM mevalonate to the medium inhibited the growth of cells harboring pMevB, this concen-

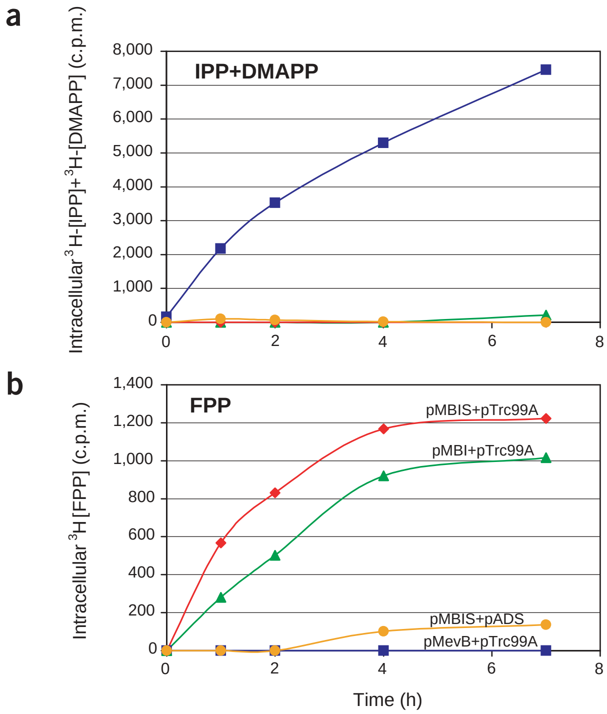

Figure 4 Prenyl pyrophosphate accumulation in resting cells harboring various mevalonate operons. (**a**) Intracellular accumulation of [^{3}H]isopentenyl pyrophosphate (IPP) and [^{3}H]dimethylallyl pyrophosphate (DMAPP). (**b**) Intracellular accumulation of [^{3}H]farnesyl pyrophosphate (FPP) from cell suspensions of *E. coli* harboring pMevB+pTrc99A, pMBI+pTrc99A, pMBIS+pTrc99A or pMBIS+pADS. The HPLC method used to analyze IPP and DMAPP could not resolve the two intermediates. Therefore, the counts per minute (c.p.m.) reported as IPP+DMAPP are from a single HPLC peak.

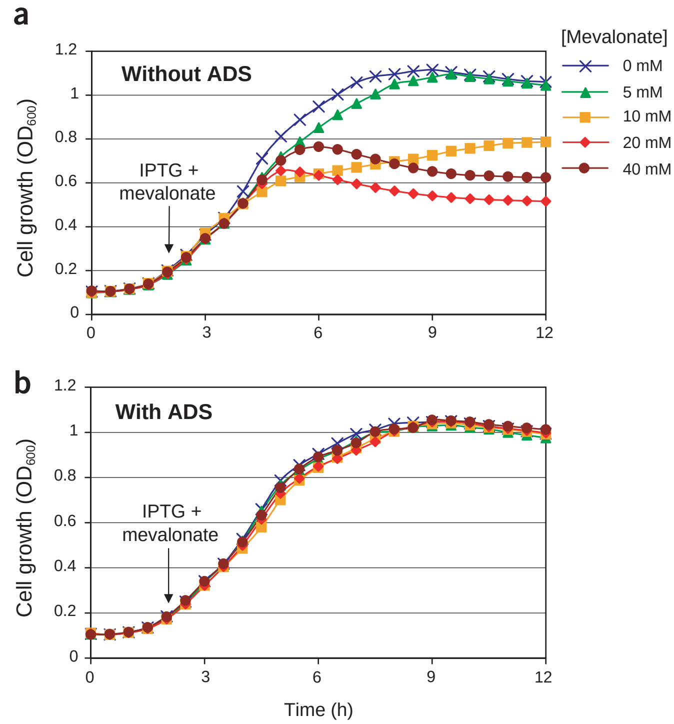

Figure 5 Effect of amorphadiene synthase (*ADS*) expression on the growth of *E. coli* harboring pMBIS. (**a**) pMBIS and the empty expression vector pTrc99A (without *ADS*) or (**b**) pADS expressing the amorphadiene synthase. LB medium was supplemented with 0 mM, 5 mM, 10 mM, 20 mM or 40 mM of DL-mevalonate.

tration of mevalonate did not affect the growth of cells harboring pMKPMK, pMBI or pMBIS (**Fig. 3**). Expression of the operons in the absence of mevalonate or in media supplemented with 1 mM mevalonate resulted in only a slight decrease in growth. Thus, from these data we hypothesized that the accumulation of IPP, which occurs in cells with high flux through the mevalonate pathway, is toxic and inhibits normal cell growth. To compare the intracellular prenyl pyrophosphate pools in the same strains, resting cells harboring the different mevalonate operon constructs were fed radiolabeled mevalonate and the labeled metabolites were tracked. As predicted, the strain expressing MevB accumulated IPP but not FPP, whereas the MBI and MBIS strains accumulated FPP but did not build up measurable levels of intracellular IPP (**Fig. 4**). Simultaneous expression of the amorphadiene synthase consumed the excess FPP pool that accumulated in the MBIS host, as shown by a decrease in intracellular FPP.

Because cells expressing MBIS accumulated FPP and exhibited growth inhibition in the presence of 10 mM mevalonate, we suspected that coexpression of the amorphadiene synthase would alleviate the growth inhibition by channeling the intracellular prenyl pyrophosphate intermediates to the volatile terpene olefin. As expected, approximately 2 h after addition of 10–40 mM mevalonate and IPTG, growth inhibition was observed only in strains lacking the *ADS* gene (**Fig. 5**). In contrast, cells coexpressing the MBIS operon and the synthase gene, both under control of IPTG-inducible promoters, exhibited normal growth rates at all mevalonate concentrations (**Fig. 5**). As shown previously, amorphadiene production from these cultures increased proportionally with the addition of exogenous mevalonate (**Fig. 2b**), further supporting the conclusion that the conversion of FPP to amorphadiene has a key role in minimizing growth inhibition. Taken together, these data strongly suggest that the engineered mevalonate pathway produces high levels of the prenyl pyrophosphate precursors. However, in the absence of the IPP isomerase, FPP synthase and terpene synthase to channel the pathway intermediates to the terpene olefin, toxic levels of intracellular prenyl pyrophosphates, especially IPP, may accumulate.

Amorphadiene synthesis from acetyl-CoA

To achieve amorphadiene production from a simple and inexpensive carbon source, the pMevT plasmid was introduced into *E. coli* harboring the pMBIS and pADS plasmids. This strain was tested for its ability to produce amorphadiene in the absence of exogenous mevalonate. Peak amorphadiene production from the complete mevalonate pathway reached 3.1 μg caryophyllene equivalent/ml/OD_{600} after 9 h of growth in LB medium. This represents 36- and 10-fold improvements over the peak production for the strains with the native and engineered DXP pathway (0.086 and 0.313 μg caryophyllene equivalents/ml/OD_{600}, respectively, as described above) (**Fig. 6**). From the comparison of the amorphadiene production between the complete (**Fig. 6**) and the 'bottom' (**Fig. 2b**) mevalonate pathways, we estimated that the MevT pathway produced the equivalent of approximately 40 mM of exogenous mevalonate. Glycerol was amended in the cultures to investigate the effect on amorphadiene yields of supplying an additional carbon source. The addition of 0.8% glycerol to the LB medium led to higher biomass yields and prolonged amorphadiene production well into the stationary phase of growth. The glycerol-amended culture reached optical densities of 3.7 and amorphadiene concentrations of 24 μg caryophyllene equivalents/ml. Using the rate of amorphadiene loss from the LB culture (**Fig. 6**) and assuming that the cells no longer produced amorphadiene after 11 h, we estimated a mass transfer coefficient of 0.87/h. By using this coefficient to account for the loss of amorphadiene to the headspace, we estimated a total production of approximately 22.6 and 112.2 mg/l from the LB and LB + 0.8% glycerol cultures, respectively. From these data, it is clear that the expression of the mevalonate-dependent isoprenoid biosynthetic pathway delivers high levels of isoprenoid precursor for the production of sesquiterpenes from a simple carbon source and that optimization of fermentation conditions should result in terpene production in the g/l range.

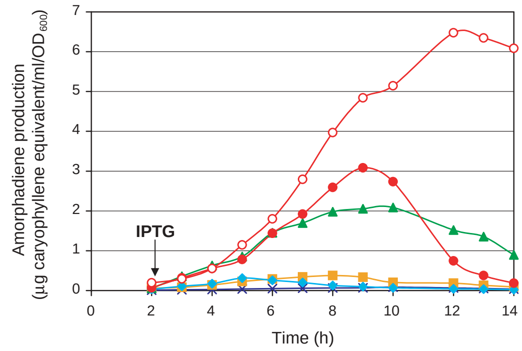

Figure 6 Comparison of amorphadiene production between *E. coli* expressing the native DXP pathway and the engineered isoprenoid pathways. The symbols represent amorphadiene production from cells supplying FPP to synthase using the native DXP pathway (pLac33, pBBR1MCS-3,dark blue ×); the engineered DXP pathway (pSOE4, pBBR1MCS-3, light blue diamond; the mevalonate bottom pathway in the absence of DL-mevalonate (pLac33, pMBIS, yellow square); the mevalonate bottom pathway in medium supplemented with 30 mM DL-mevalonate (pLac33, pMBIS, green triangle); the complete mevalonate pathway (pMevT, pMBIS, filled red circle) and the complete mevalonate pathway in medium supplemented with 0.8% glycerol (open red circle).

DISCUSSION

The development of artemisinin, a promising and potent antimalarial drug, has been limited by the costs associated with extracting the compound from its natural sources and the complexity of the alternative chemical synthesis[2,7,24]. Classical plant breeding and selection combined with improved agricultural practices may not be adequate to lower the costs of artemisinin production to a price affordable for those most affected by the pathogen. Several laboratories have focused on the isolation of *Artemisia annua* L. genes involved in artemisinin synthesis in the hope of lowering the cost of artemisinin production by improving the yields from genetically engineered plants[20,25–27]. The first gene discovered encoded the amorphadiene synthase, which converts FPP to amorphadiene. We sought to capitalize on this discovery by expressing the terpene synthase gene in a microbial host engineered to produce high levels of the FPP precursor for enhanced yields of the sesquiterpene olefin.

From our previous work on the microbial production of plant sesquiterpenes in *E. coli*, we concluded that poor expression of the synthase genes limits high terpene olefin yields from this host[21]. To circumvent this limitation, we chose to synthesize an amorphadiene synthase gene from oligonucleotides using the *E. coli* codon preferences, which can greatly improve protein expression[28]. Comparison of sesquiterpene production in *E. coli* expressing native sesquiterpene synthase genes[21] and *E. coli* expressing the synthetic *ADS* gene showed from a 10- to 300-fold improvement in terpene synthesis in the latter.

Insufficient supply of the prenyl pyrophosphate precursor by the native DXP pathway was shown to limit carotenoid[29] and taxadiene[30] yields in *E. coli*. Metabolic engineering of the DXP pathway[17,18,31–34] increased the flux in carotenoid accumulation by 2- to 40-fold over incubation periods of 20–50 h. Likewise, in this study we observed a threefold increase in sesquiterpene accumulation after 5 h using a similar engineering strategy (**Fig. 6**). These observations imply that this approach to engineering the DXP pathway results in only a modest increase in flux that may be detectable only by using carotenoid biosynthesis as a reporter and long incubation periods. In this work, we demonstrate that the mevalonate isoprenoid pathway is a superior biosynthetic route for delivering high-level isoprenoid precursors to terpene synthases for large-scale production. By engineering the *S. cerevisiae* mevalonate-dependent isoprenoid pathway into *E. coli*, we circumvent the mevalonate pathway's native regulatory elements found in yeast while bypassing those of *E. coli*'s native DXP pathway. In fact, the heterologous pathway leads to such a vast excess of prenyl pyrophosphates that cell growth is inhibited. Coexpression of a synthetic sesquiterpene synthase consumes the excess pool of precursor, thereby eliminating growth inhibition and providing high yields of amorphadiene. Although total biosynthesis of artemisinin was not achieved in this study, the engineered biochemical pathway could be extended to produce artemisinic acid. Artemisinic acid can then be converted to high yields (40%) of artemisinin or one of the derivatives via a photo-oxidation cyclization reaction[35].

In summary, the production of terpene-based compounds first requires the ability to produce large quantities of the olefin precursor. Our work provides a microbial host capable of producing precursors for the large-scale production of any terpene olefin and represents the first essential step toward the production of a broad range of terpene-based compounds in microorganisms. The use of microbes as platform hosts for the synthesis of terpenes offers several advantages over existing methods because they are better suited for the engineering of enzymes and biochemical pathways. For example, the amorphadiene gene may be easily replaced with any terpene synthase for high-level production of the new terpene. Furthermore, *in vitro* evolution and combinatorial biosynthesis of sesquiterpene biochemical pathways in microbes may lead to artemisinin derivatives or even new sesquiterpene lead compounds. Although greatly improved yields were obtained by combining the expression of a synthetic sesquiterpene synthase with a recombinant mevalonate pathway, the data suggest that a maximum yield was not attained. Therefore, efforts are now directed at identifying the pathway bottlenecks to maximize the flux and optimize expression of the mevalonate pathway.

METHODS

Strains and media. *E. coli* DH10B was used as the cloning and isoprenoid expression strain (see **Supplementary Table 1** online for a summary of the strains and plasmids used in this study). For the growth studies, the optical density of cultures expressing the various recombinant pathways was measured with a microtiter plate reader (SpectraMax, Molecular Devices) from 200 μl cultures of LB broth in 96-well plates incubated at 37 °C with continuous shaking. DL-Mevalonolactone was purchased from Sigma-Aldrich and 2-*C*-methyl-D-erythritol was synthesized from citraconic anhydride according to the protocol of Duvold *et al.*[36]. The *isp*C mutant *E. coli* strain DYM1[22] (kindly provided by Haruo Seto, University of Tokyo) was used to test the functionality of the synthetic mevalonate operons. The DYM1 strain was propagated on LB medium containing 0.5 mM methylerythritol and transformed DYM1 cells were first allowed to recover on plates supplemented with methylerythritol before being streaked on test media. Media used to test the functionality of the operons were supplemented with 1 mM DL-mevalonate prepared by mixing 1 volume of 2 M DL-mevalonolactone with 1.02 volumes of 2 M KOH and incubating at 37 °C for 30 min[37].

Synthesis of amorphadiene synthase gene. The synthetic *ADS* gene was designed using Calcgene[28] and the protein sequence of the synthase isolated by Mercke *et al.*[20] (see **Supplementary Fig. 1** online for gene sequence and list of oligonucleotides). To assemble the *ADS* gene, each of the 84 overlapping oligonucleotides (Gibco-BRL) was dissolved in distilled, deionized H_2O to a final concentration of 100 µM. A mixture was prepared by combining 10 µl of each of the individual oligonucleotides. The first PCR reaction in the two-step PCR assembly of *ADS* contained in 100 µl, 1× *Pfu* polymerase buffer (20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1% mg/ml BSA), 0.25 mM of each dNTP, 1 µM of oligonucleotides mixture and 5 U *Pfu* polymerase (Stratagene). The PCR cycling program was 94 °C for 30 s, 40 °C for 2 min, 72 °C for 10 s followed by 40 cycles at 94 °C for 15 s, 40 °C for 30 s, 72 °C for 20 s + 3 s per cycle. The second PCR reaction contained in 100 µl, 33 µl of the first assembly reaction, 1× *Pfu* buffer, 0.25 mM of each dNTP and 5U *Pfu* polymerase. The PCR program for the second step of the assembly was as follows: 94 °C for 30 s, 40 °C for 10 s, 72 °C for 10 s followed by 25 cycles at 94 °C for 15 s, 40 °C for 30 s, 72 °C for 45 s + 1 s per cycle. The DNA smear in the range of 1.7 kb was gel purified and used as template for a third and final PCR reaction containing in 100 µl, 1X *Pfu* buffer, 0.25 mM of each dNTP, 250 nM each of the two outside primers (T-1 and B-42), 10 µl of the gel purified DNA and 5 U *Pfu* polymerase. The PCR program was 40 cycles of 94 °C for 45 s and 72 °C for 4 min followed by a final step at 72 °C for 10 min. The expected 1.7-kb band was gel purified and ligated into pTrc99A using 5′ *Nco*I and 3′ *Xma*I sites designed into the gene sequence, thereby generating pADS. Two rounds of site-directed mutagenesis were needed to eliminate point mutations and generate a functional gene.

Construction of the DXP pathway operon. The *dxs* gene of *E. coli* was spliced to the IPP isomerase gene (*ippHp*) from pAC-LYC04 (ref. 38) using overlapping extensions and PCR primers dxs1, dxs2, ippHp1 and ippHp2 (see **Supplementary Table 2** online for primer sequences). The *E. coli ispA* gene was isolated by PCR using primers ispa1 and ipsa2 and ligated to the *Nco*I site 3′ to *ippHp*. The three-gene DXP operon was amplified with primers SOE-f and ispa2 and ligated into the *Kpn*I-*Pst*I sites of pMevT, thereby replacing the MevT operon with the SOE4 operon.

Construction of the mevalonate pathway operons. The *S. cerevisiae* mevalonate pathway was engineered as two separate, independently expressed operons. The genes encoding the last three enzymes of the biosynthetic pathway, mevalonate kinase (MK; gene, *ERG12*), phosphomevalonate kinase (PMK; gene, *ERG8*) and mevalonate pyrophosphate decarboxylase (MPD; gene, *MVD1*, also known as *ERG19*), were isolated by PCR from chromosomal DNA preparations of *S. cerevisiae*. The individual genes were spliced together (MevB, **Fig. 1**) using overlapping extensions from primers MK-f, MK-r, PMK-f, PMK-r, MPD-f and MPD-r. The genes encoding the first three enzymes of the mevalonate pathway, the acetoacetyl-CoA thiolase from *E. coli* (AACT or *ato*B), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS or *ERG13*) and a truncated version of 3-hydroxy-3-methylglutaryl-CoA reductase[39] (tHMGR1), were isolated and spliced together as a single operon (MevT, **Fig. 1**) using the following primers: atoB-f, atoB-r, HMGS-f, HMGS-r, tHMGR-f and tHMGR-r. Individual genes were isolated by PCR using *Pfu* DNA polymerase and a standard PCR protocol. The synthetic operons were ligated into pCR4 (TA vector from Invitrogen), after the addition of 3′ A overhangs, and sequenced to ensure accuracy. The MevB operon was ligated into the *Pst*I site of pBBR1MCS-1 (ref. 40), generating pMevBCm. The *idi* gene was ligated into the *Xma*I site, 3′ to MevB using primers idi-f and idi-r and the MBI operon was moved to the *Sal*I-*Sac*I sites of pBBR1MCS-3 to generate pMBI. The *idi* gene was excised from pMBI using *Xma*I, thereby generating pMevB. The *isp*A gene from *E. coli* was ligated into the *Sac*I-*Sac*II sites of pMBI using primers ispa-f and ispa-r, thereby producing pMBIS. The MevT operon was ligated into the *Xma*I-*Pst*I sites of pBAD33 (ref. 41). To place the operon under control of the P_{LAC} promoter, the *araC*-P_{BAD} *Nsi*I-*Xma*I fragment was replaced with the *Nsi*I-*Xma*I fragment of pBBR1MCS, thereby generating pMevT. To generate pLac33, the MevT operon was excised from pMevT with *Sal*I.

GC-MS analysis of amorphadiene. Amorphadiene production by the various strains was measured by GC-MS as previously described[21] by scanning only for two ions, the molecular ion (204 *m/z*) and the 189 *m/z* ion. Cells were grown in LB medium at 37 °C for 2 h and induced to express the *ADS* and the mevalonate pathway by the simultaneous addition of 0.5 mM IPTG and varying concentrations of mevalonate. 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. The sesquiterpene caryophyllene was purchased from Sigma-Aldrich.

Radio-HPLC analysis of intracellular prenyl pyrophosphates. Intracellular IPP+DMAPP and FPP levels were measured using a resting cell suspension assay supplemented with *(R)*-[5-^{3}H]mevalonate (39 Ci/mmol; Perkin-Elmer Life Sciences). Cells induced with 0.5 mM IPTG were grown in LB broth at 37 °C to an OD_{600} of ~0.6, harvested, washed once and suspended to 20× concentration in 100 mM KPO_4 buffer (pH 7.4). Unlabeled DL-mevalonate (10 mM) and ^{3}H-radiolabeled *(R)*-mevalonate (60 µCi) were added to 8 ml of cell suspension and incubated at 37 °C. Cells from 1.5-ml aliquots were washed twice with cold KPO_4 buffer and the intracellular IPP+DMAPP and FPP were extracted from cell pellets with 1 ml of 2:1 (vol/vol) methanol/chloroform. The cell extracts were dephosphorylated using potato acid phosphatase as previously described by Fujii *et al.*[42] The prenyl alcohols were resolved on a reverse phase C-18 column (4.5 mm × 250 mm, 5 µm particle size; Alltech) by HPLC (Agilent Technologies model 1100) using the method of Zhang and Poulter[43] and detected with a flow-through scintillation counter (Packard BioScience, Radiomatic model 500TR).

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS
The authors would like to acknowledge Seon-Won Kim, Stephen del Cardayré, Ranjini Chaterjee and David Williams for their contributions and Ahamindra Jain for the synthesis of 2-*C*-methyl-D-erythritol. This work was supported by research grants from the US National Science Foundation (grant number BES-9911463), University of California BioSTAR (grant number 99-10044), Maxygen and the Office of Naval Research (grant number FDN00014-99-0182). D.J.P. is the recipient of a National Science Foundation graduate fellowship.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

Received 21 November 2002; accepted 17 March 2003
Published online 1 June 2003; doi:10.1038/nbt833

1. Cragg, G.M. Paclitaxel (Taxol): a success story with valuable lessons for natural product drug discovery and development. *Med. Res. Rev.* **18**, 315–331 (1998).
2. Dhingra, V., Rao, K.V. & Narasu, M.L. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci.* **66**, 279–300 (2000).
3. Danishefsky, S.J. *et al.* Total synthesis of baccatin III and taxol. *J. Amer. Chem. Soc.* **118**, 2843–2859 (1996).
4. Nicolaou, K.C. *et al.* Total synthesis of eleutherobin. *Angew. Chem. Int. Ed.* **36**, 2520–2524 (1997).
5. Avery, M.A., Chong, W.K.M. & Jennings-White, C. Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. *J. Amer. Chem. Soc.* **114**, 974–979 (1992).
6. White, N.J. Artemisinin—Current status. *Trans. R. Soc. Trop. Med. Hyg.* Suppl. **88**, 53–54 (1994).
7. Ridley, R.G. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature* **415**, 686–693 (2002).
8. Haynes, R.K. Artemisinin and derivatives: the future for malaria treatment? *Curr. Opin Infect. Dis.* **14**, 719–726 (2001).
9. Wallaart, T.E., Pras, N., Beekman, A.C. & Quax, W.J. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Med.* **66**, 57–62 (2000).
10. Jennewein, S. & Croteau, R. Taxol: biosynthesis, molecular genetics, and biotechnological applications. *Appl. Microbiol. Biotechnol.* **57**, 13–19 (2001).
11. Skeel, R.T. *Handbook of Cancer Chemotherapy*, edn. 5 (Lippincott Williams & Wilkins, Philadelphia, 1999).
12. Baekelandt, M. Irofulven (MGI Pharma). *Curr. Opin. Investig. Drugs* **3**, 1517–1526 (2002).
13. Amato, R.J., Perez, C. & Pagliaro, L. Irofulven, a novel inhibitor of DNA synthesis, in metastatic renal cell cancer. *Invest. New Drugs* **20**, 413–417 (2002).
14. Boucher, Y. & Doolittle, W.F. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol. Microbiol.* **37**, 703–716 (2000).
15. Rohdich, F. *et al.* Studies on the nonmevalonate terpene biosynthetic pathway: meta-

bolic role of IspH (LytB) protein. *Proc. Natl. Acad. Sci. USA* **99**, 1158–1163 (2002).

16. Connolly, D.M. & Winkler, M.E. Genetic and physiological relationships among the *miaA* gene, 2-methylthio-N6-(Δ2-isopentenyl)adenosine transfer RNA modification, and spontaneous mutagenesis in *Escherichia coli* K-12. *J. Bacteriol.* **171**, 3233–3246 (1989).
17. Farmer, W.R. & Liao, J.C. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog.* **17**, 57–61 (2001).
18. Kajiwara, S., Fraser, P.D., Kondo, K. & Misawa, N. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.* **324**, 421–426 (1997).
19. Kim, S.-W. & Keasling, J.D. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng.* **72**, 408–415 (2001).
20. Mercke, P., Bengtsson, M., Bouwmeester, H.J., Posthumus, M.A. & Brodelius, P.E. Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Arch. Biochem. Biophys.* **381**, 173–180 (2000).
21. Martin, V.J.J., Yoshikuni, Y. & Keasling, J.D. The *in vivo* synthesis of plant sesquiterpenes by *Escherichia coli*. *Biotechnol. Bioeng.* **75**, 497–503 (2001).
22. Kuzuyama, T., Takahashi, S. & Seto, H. Construction and characterization of *Escherichia coli* disruptants defective in the yaeM gene. *Biosci. Biotechnol. Biochem.* **63**, 776–778 (1999).
23. Hahn, F.M., Hurlburt, A.P. & Poulter, C.D. *Escherichia coli* open reading frame 696 is idi, a nonessential gene encoding isopentenyl diphosphate isomerase. *J. Bacteriol.* **181**, 4499–4504 (1999).
24. Van Geldre, E., Vergauwe, A. & Van den Eeckhout, E. State of the art of the production of the antimalarial compound artemisinin in plants. *Plant Mol. Biol.* **33**, 199–209 (1997).
25. Bouwmeester, H.J. *et al.* Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochem.* **52**, 843–854 (1999).
26. Wallaart, T.E., Bouwmeester, H.J., Hille, J., Poppinga, L. & Maijers, N.C.A. Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta* **212**, 460–465 (2001).
27. Chang, Y.J., Song, S.H., Park, S.H. & Kim, S.U. Amorpha-4,11-diene synthase of *Artemisia annua*: cDNA isolation and bacterial expression of a terpene synthase involved in artemisinin biosynthesis. *Arch. Biochem. Biophys.* **383**, 178–184 (2000).
28. Hale, R.S. & Thompson, G. Codon optimization of the gene encoding a domain from human type 1 neurofibromin protein results in a threefold improvement in expression level in *Escherichia coli*. *Protein Exper. Purif.* **12**, 185–188 (1998).
29. Sandmann, G. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chembiochem.* **3**, 629–635 (2002).
30. Huang, Q.L., Roessner, C.A., Croteau, R. & Scott, A.I. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorgan. Med. Chem.* **9**, 2237–2242 (2001).
31. Matthews, P.D. & Wurtzel, E.T. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. *Appl. Microbiol. Biotechnol.* **53**, 396–400 (2000).
32. Albrecht, M., Misawa, N. & Sandmann, G. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechnol. Lett.* **21**, 791–795 (1999).
33. Harker, M. & Bramley, P.M. Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett.* **448**, 115–119 (1999).
34. Wang, C.-W., Oh, M.-K. & Liao, J.C. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol. Bioeng.* **62**, 235–241 (1999).
35. Jung, M., ElSohly, H.N. & McChesney, J.D. Artemisinic acid: a versatile chiral synthon and bioprecursor to natural products. *Planta Med.* **56**, 624 (1990).
36. Duvold, T., Bravo, J.M., Pale-Grosdemange, C. & Rohmer, M. Biosynthesis of 2-*C*-methyl-D-erythritol, a putative C-5 intermediate in the mevalonate independent pathway for isoprenoid biosynthesis. *Tetrahedron Lett.* **38**, 4769–4772 (1997).
37. Campos, N. *et al. Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. *Biochem. J.* **353**, 59–67 (2001).
38. Cunningham, F.X., Sun, Z., Chamovitz, D., Hirschberg, J. & Gantt, E. Molecular structure and enzymatic function of lycopene cyclase from the *Cyanobacterium synechococcus* sp. strain PCC7942. *Plant Cell* **6**, 1107–1121 (1994).
39. Polakowski, T., Stahl, U. & Lang, C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. *Appl. Microbiol. Biotechnol.* **49**, 66–71 (1998).
40. Kovach, M.E. *et al.* Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176 (1995).
41. Guzman, L.-M., Belin, D., Carson, M.J. & Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**, 4121–4130 (1995).
42. Fujii, H., Koyama, T. & Ogura, K. Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. *Biochem. Biophys. Acta* **712**, 716–718 (1982).
43. Zhang, D.L. & Poulter, C.D. Analysis and purification of phosphorylated isoprenoids by reversed-phase HPLC. *Anal. Biochem.* **213**, 356–361 (1993).