

Mono and Diterpene Production in *Escherichia coli*

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Abstract: Mono- and diterpenoids are of great industrial and medical value as specialty chemicals and pharmaceuticals. Production of these compounds in microbial hosts, such as *Escherichia coli*, can be limited by intracellular levels of the polyprenyl diphosphate precursors, geranyl diphosphate (GPP), and geranylgeranyl diphosphate (GGPP). To alleviate this limitation, we constructed synthetic operons that express three key enzymes for biosynthesis of these precursors: (1) DxS, 1-deoxy-D-xylulose-5-phosphate synthase; (2) IPIHp, IPP isomerase from *Haematococcus pluvialis*; and (3) one of two variants of IspA, FPP synthase that produces either GPP or GGPP. The reporter plasmids pAC-LYC and pACYC-IB, which encode enzymes that convert either FPP or GGPP, respectively, to the pigment lycopene, were used to demonstrate that at full induction, the operon encoding the wild-type FPP synthase and mutant GGPP synthase produced similar levels of lycopene. To synthesize di- or monoterpenes in *E. coli* using the GGPP and GPP encoding operons either a diterpene cyclase [casbene cyclase (*Ricinus communis* L) and *ent*-kaurene cyclase (*Phaeosphaeria* sp. L487)] or a monoterpene cyclase [3-carene cyclase (*Picea abies*)] was coexpressed with their respective precursor production operon. Analysis of culture extracts or headspace by gas chromatography-mass spectrometry confirmed the *in vivo* production of the diterpenes casbene, kaur-15-ene, and kaur-16-ene and the monoterpenes α -pinene, myrcene, sabinene, 3-carene, α -terpinene, limonene, β -phellandrene, α -terpinene, and terpinolene. Construction and functional expression of GGPP and GPP operons provides an *in vivo* precursor platform host for the future engineering of di- and monoterpene cyclases and the overproduction of terpenes in bacteria. © 2004 Wiley Periodicals, Inc.

Keywords: isoprenoid; diterpene; monoterpene; cyclase; casbene; carene; kaurene

INTRODUCTION

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems; it has been estimated that there are over 50,000 known isoprenoids, which include the terpenoids and carotenoids (McCaskill and Croteau, 1997). The number of isoprene (C₅) units of which they are comprised classifies terpenoids. Monoterpenes (C₁₀), such as menthol and camphor, and sesquiterpenes (C₁₅), such as zingiberene (ginger), are the major constituents of herbs and spices. Other sesquiterpenes and diterpenes (C₂₀) are pheromones, antimicrobial agents, or signal transducers (Fraga, 1991; McGarvey and Croteau, 1995). Higher molecular weight isoprenoids stabilize membranes (cholesterol and other C₃₀ compounds) and serve as photoreceptive pigments (lycopene).

The primary or ancillary biological activity of some terpenoids has been a benefit to the medical community. Several monoterpene derivatives are being investigated as anticancer agents. For example, the monoterpene limonene and related derivatives are believed to inhibit farnesylation of the growth-promoting protein RAS, and therefore inhibit malignant cell proliferation (Gelb et al., 1995; Gould, 1997; Hohl, 1996). Casbene, a plant diterpenoid, is an antifungal agent (Erkel and Anke, 1994; Ferrandiz et al., 1994; Tan et al., 1999). Taxol, a diterpenoid isolated from the pacific yew tree (*Taxus brevifolia*), and its derivatives are potent anti-cancer compounds used in the treatment of ovarian, breast, lung, neck, bladder, skin, and cervical cancers (Jennewein and Croteau, 2001). Because the complexity of Taxol precludes commercial total chemical synthesis (Jennewein and Croteau, 2001) nearly all of the Taxol

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biosynthetic genes have been cloned, opening the way for in vivo recombinant production of a diterpene therapeutic.

The building blocks for the biosynthesis of terpenes, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP), can be synthesized via two pathways: the mevalonate-dependent and the mevalonate-independent pathway (also referred to as the DXP pathway; Fig. 1). In

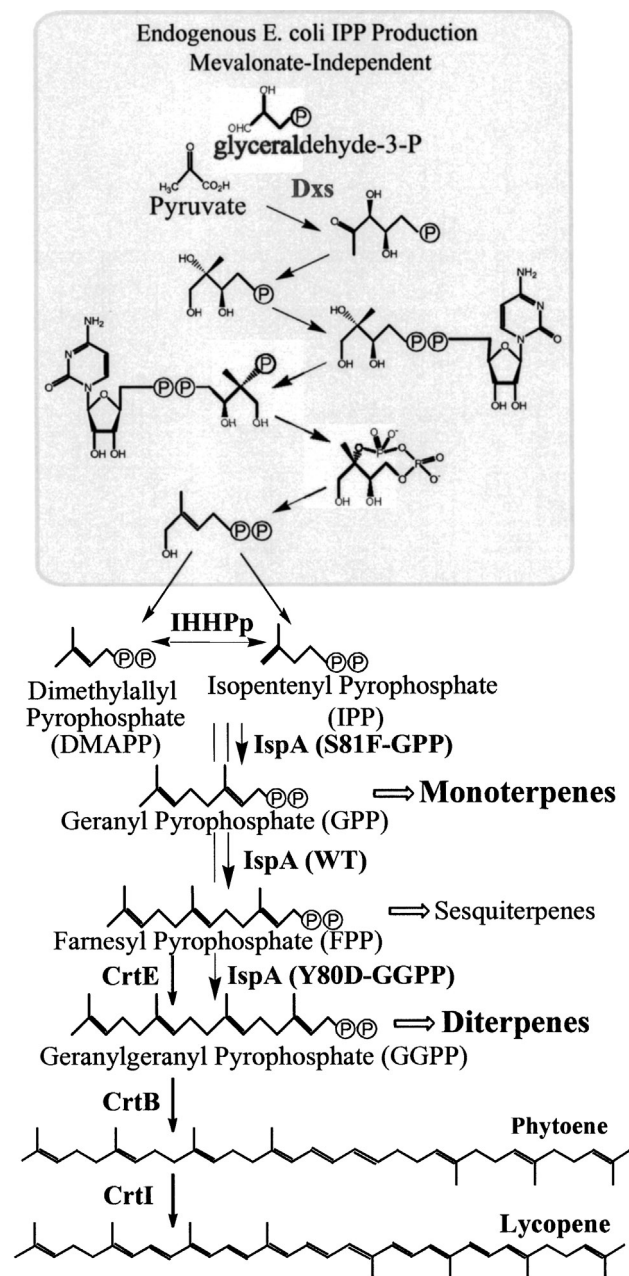


Figure 1. The DXP pathway and downstream compounds addressed in this study. In the upper box is depicted the metabolite flow leading to IPP through the DXP pathway of *E. coli*. Below is a flow of metabolites leading to either terpenes or the pigment lycopene. Listed are the enzymes used in this study: in the SOE operons (DXP synthase, *dxs*; FPP synthase, *ispA*; IPP isomerase, *IPHh*), in the pAC-LYC reporter plasmids (GGPP synthase, *crtE*; phytoene synthase, *crtB*; and lycopene synthase, *crtI*) and in the pACYC-IB (*crtB* and *crtI*).

prokaryotes, isoprenoids are synthesized primarily via the DXP pathway (Boucher and Doolittle, 2000), which in *Escherichia coli* produces farnesyl diphosphate (FPP). Farnesyl diphosphate is the precursor to *trans*-octaprenyl diphosphate, used in ubiquinone synthesis, and *cis*, *trans*-undecaprenyl diphosphate, used in bactoprenol biosynthesis (Apfel et al., 1999). *Escherichia coli*, however, does not naturally produce appreciable quantities of geranyl diphosphate (GPP) or geranylgeranyl diphosphate (GGPP), the precursors for mono- and diterpenes, respectively.

In this study, we have developed strains of *E. coli* that harbor engineered operons encoding enzymes for the production of GPP and GGPP. The output of the GGPP biosynthetic pathway is assayed via the production of the pigment lycopene from these precursors. We report on the in vivo production of mono- and diterpenes from GPP or GGPP by three terpene cyclases: the monoterpene synthase 3-carene cyclase from *Picea abies* (Faldt et al., 2003), and the diterpene synthases casbene cyclase from *Ricinus communis* L (Mau and West, 1994) and *ent*-kaurene cyclase from *Phaeosphaeria* sp. L487 (Kawaide et al., 1997).

The terpene-precursor platform host, described in this study, is designed to facilitate the transition of natural products from discovery to development and commercialization. Terpenes and their derivatives are often difficult to produce in significant yields either in higher plants or by synthetic means. Although the research on metabolic engineering of natural product biosynthesis is still at the gene discovery stage, the deluge of DNA-sequence data from genomics research provides an excellent opportunity for the design of microorganisms for the synthesis of natural products. Combined with recent advances in enzyme directed evolution and combinatorial biocatalysis, this approach may be a valuable alternative to traditional methods of high-throughput small-molecule synthesis and drug production.

MATERIALS AND METHODS

Media and Culture

Luria-Bertani (LB), M9, and 2YT media were used in this study (Sambrook et al., 1989). Carbenicillin (50 $\mu\text{g/mL}$), kanamycin (50 $\mu\text{g/mL}$), ampicillin (100 $\mu\text{g/mL}$), chloramphenicol (50 $\mu\text{g/mL}$), isopropyl-beta-D-thiogalactopyranoside (IPTG), and arabinose were purchased from Fisher Scientific. Plasmids used in this study are listed in Table I.

Construction and Expression of the Synthetic Isoprenoid Operons

The synthetic operons were constructed by splicing the genes using overlapping extensions (SOE). These operons expressed a combination of three genes: the *E. coli* 1-deoxy-D-xylulose-5-phosphate synthase gene (*dxs*-AC# NP_414954; Blattner et al., 1997), the *Haematococcus*

Table I. Plasmids used in this study.

Vector	Parent plasmid	Antibiotic marker	Expression control	Inserted gene	Reference
pTRcCas	pTrc99a	Carbenicillin	LacIq	Casbene Cyclase	This study
pTRcKau	pTrc99a	Carbenicillin	LacIq	<i>ent</i> -Kaurene Cyclase	This study
pTRcCar	pTrc99a	Carbenicillin	LacIq	Carene Cyclase	This study
pBADSOE1	pBAD24	Carbenicillin	AraC	<i>dxs</i> , <i>IPIHp</i>	This study
pBADSOE4	pBAD24	Carbenicillin	AraC	<i>dxs</i> , <i>IPIHp</i> , <i>ispA</i> (WT)	This study
pBBRSOE4	pBBR1MCS-2	Kanamycin	AraC	<i>dxs</i> , <i>IPIHp</i> , <i>ispA</i> (WT)	This study
pBBRSOE5	pBBR1MCS-3	Kanamycin	AraC	<i>dxs</i> , <i>IPIHp</i> , <i>ispA</i> (Y80D)	This study
pBBRSOE6	pBBR1MCS-4	Kanamycin	AraC	<i>dxs</i> , <i>IPIHp</i> , <i>ispA</i> (S81F)	This study
pAC-LYC	pACYC154	Chloramphenicol	Constitutive	<i>crtE</i> , <i>crtB</i> , <i>crtI</i>	Cunningham et al., 1994
pACYC-IB	pACYC154	Tetracycline	Constitutive	<i>crtB</i> , <i>crtI</i>	Ohnuma et al., 1996

pluvialis isopentenyl diphosphate (IPP) isomerase gene (*IPIHp*-AC# AF082326; Sun et al., 1998), and the *E. coli* FPP synthase gene (*ispA*-AC# NP_414955; Blattner et al., 1997). *dxs* was obtained from the plasmid pDdxs (Kim and Keasling, 2001). *IPPHp* was subcloned from the previously constructed plasmid, pAC-LYC04 (Cunningham et al., 1994). *IPPHp* was spliced 3' to *dxs* via PCR using overlap extensions and primers *dxs*-for and *dxs*-rev and *IPPHp*-for and *IPPHp*-rev (Table II). The *dxs*-for primer introduced a 5'-*Eco*RI site upstream of the two-gene operon and the *IPPHp*-rev primer introduced a 3'-*Xba*I site downstream. The overlapping *dxs*-rev and *IPPHp*-for primers introduced a Shine-Dalgarno site in place of the final two codons of the *dxs* gene changing the N-terminal sequence from Leu-Ala (GGCTGGCATAA) to Arg-Thr (GGAGGACATAA).

Thus, the 10-base spacer between the Shine-Dalgarno site and the start codon of *IPPHp* required only a four base separation between the two genes. The variant *ispA* genes (isolated from the pCRisA plasmids, described below) were spliced to the 3' end of the *dxs*-*IPPHp* operon by the introduction of a 3'-*Nco*I site flanking the *dxs*-*IPPHp* fragment via PCR using the primers *dxs*-for2 and *IPPHp*-rev2. The *dxs*-for2 primer introduced a 5'-*Nhe*I site and a Shine-Dalgarno site into the *dxs*-*IPPHp* fragment. The *IPPHp*-rev2 primer mutated the two C-terminal residues of *IPPHp* from Glu-Ala (GCGTGA) to Gly-Gly (GGAGGA) and thus introduced a Shine-Dalgarno site seven base pairs 5' to the ATG of the *Nco*I site. This *dxs*-*IPPHp* PCR product was ligated into the *Nhe*I-*Nco*I sites of the pBAD24 plasmid to produce pBADSOE1, which was subsequently

Table II. PCR primers used in this study.

Primer	Sequence ^a
S81F-for	5'-GTGTATCCACGCTTACTTTTAAATTCATGATGATT-3'
S81F-rev	5'-AATCATCATGAATTAAGTAAGCGTGGATACAC-3'
Y80D-for	5'-TGAGTGTATCCACGCTGACTCATTAATTCATGATG-3'
Y80D-rev	5'-CATCATGAATTAATGAGTCAGCGTGGATACACTCA-3'
	<i>Eco</i> RI
<i>dxs</i> -for	5'-AGGAGGAATTCACCATGAGTTTGTATTTGCCAAATAC-3'
<i>dxs</i> -rev	5'-TCTGAGCAACGAACGAAGCATATATTTATGTCTCCAGGCCTTG ATTTTG-3'
<i>IPPHp</i> -for	5'-CAAAATCAAGGCCTGGAGGACATAAATATATGCTTCGTTCTGTTG CTCAGA-3'
<i>IPPHp</i> -rev	5'-GCTCTAGATCAGCCTTCGTTGATGTGATGC-3'
	<i>Xba</i> I
	<i>Nhe</i> I <i>Eco</i> RI
<i>dxs</i> -for2	5'-TTGGGCTAGCAGGAGGAATTC-3'
<i>IPPHp</i> -rev2	5'-GCATCCATGGTATCATCCTCCGTTGATGTGATG-3'
	<i>Nco</i> I
	<i>Nco</i> I
<i>ispA</i> -for	5'-TGATACCATGGACTTTCCGACGAACTCG-3'
<i>ispA</i> -rev	5'-GCTCTAGATTATTTATTACGCTGGATGATG-3'
	<i>Xba</i> I
	<i>Xba</i> I
Kaurene-for	5'-GCTCTAGAATGTTTGCCAAATTCGATATGC-3'
Kaurene-rev	5'-CCCAAGCTTTTACGTGCCGACGTGTTTCAAAG-3'
	<i>Hind</i> III
	<i>Nco</i> I
3CNeoF	5'-TAGCCATGGCTGTTATTTCCATTTTGCCGTTG-3'
3CXbaR	5'-GCTCTAGATTACATAGGCACAGGTTCAAGAACGG-3'

^a*Italics* represent the Shine-Dalgarno (SD) sequences, underlined text denotes the restriction site sequences, and **bold** type indicates the start/stop codons.

sequenced. The *ispA* gene or its variants were amplified from the pCRispA vectors described below using the PCR primers *ispA*-for and *ispA*-rev, which introduced an *NcoI* site in frame with the start codon and an *XbaI* site immediately after the stop codon. The produced fragments were ligated into the multiple cloning site of the pBAD24 vector, which contains the arabinose-inducible *araBAD* promoter, to yield pBADSOE4, pBADSOE5, or pBADSOE6 (Fig. 2). The *ispA* region of each of the pBADSOE operons were subsequently sequenced in the context of the pET30a expression vectors (described below) to confirm the integrity of the gene. The operon fragments generated by a *Clal-XbaI* digest were ligated into the broad-host-range vector pBBR1MCS-2 to produce pBBRSOE4, pBBRSOE5, or pBBRSOE6. The fragments generated by this digest contained the prenyl diphosphate biosynthetic operon, the arabinose-inducible P_{BAD} and associated regulator (*araC*). All the PCR reactions were run for 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min and used Expand High Fidelity PCR System (Roche).

Cloning of Mono- and Diterpene Cyclases

The terpene cyclases used in this study were the 3-carene cyclase from *Picea abies* (AC# AF461460), the *ent*-kaurene cyclase from *Phaeosphaeria* sp. L487 (AC# AB003395), and the casbene cyclase from *Ricinus communis* L (AC# L32134). The casbene cyclase cDNA, initially provided in a pET21d plasmid, was ligated into pTrc99A at the *NcoI* and *SalI* restriction sites to generate pTrcCAS. The *ent*-kaurene cyclase gene was amplified from plasmid DNA and 5'-*XbaI* and 3'-*HindIII* restriction sites were introduced by PCR using the primers kaurene-for and kaurene-rev, and the Expand HF PCR system (Roche). This PCR fragment was digested and ligated into the *XbaI-HindIII* sites of pTrc99A to produce pTrcKau. Carene cyclase was amplified from plasmid DNA, and a 5'-*NcoI* site and a 3'-*XbaI* site were introduced via PCR using the primers 3CNcoF and 3CXbaR (Table II). The resulting PCR fragment was ligated into the like restriction sites of pTrc99a to yield pTrcCar. The sequence of the cyclase genes amplified by PCR was confirmed by DNA sequencing. Each of the sequenced clones of carene exhibited the silent mutation C → A at base pair 561. The cyclase plasmids were

cotransformed into either *E. coli* DH5α (pTrcKau) or DH10b (pTrcCas and pTrcCar) with the appropriate isoprenoid operon for in vivo terpene production.

Production and Purification of Wild-Type and Mutant *ispA* Prenyltransferases

Wild-type and mutant prenyltransferase genes were expressed from a pET30a vector and the 6-His-tagged recombinant enzymes purified by immobilized-metal affinity chromatography (IMAC). The *ispA* genes were subcloned into a pET30a vector from the SOE operons using PCR to introduce 5'-*NcoI-EcoRI*-3' restriction sites (Table II), sequenced, and transformed into *E. coli* BL21(DE3) (Novagen) for protein production. Overnight cultures were grown from a single colony and used to inoculate (1% v/v) 500 mL of LB medium. The cultures were grown at 37°C to an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.6 at which time they were induced with 0.1 mM IPTG. Cells were harvested 4 h post-induction and the pellets were frozen at -80°C. Cells harvested from 500 mL of culture were suspended in 5 mL of 50 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF, DNaseI, 10 mM $CaCl_2$, and 400 mM NaCl (buffer A) and lysed on ice by three successive sonications using a 50% duty cycle at 25% power for 1 min. Lysates were cleared by centrifugation at 31,400 RCF and 4°C for 30 min and supernatants were filtered through a 0.2 μm syringe filter. The cleared lysates were loaded at 1 mL/min onto a 1 mL fast flow chelating sepharose resin (Pharmacia) previously charged with 100 mM $NiSO_4$ and equilibrated with buffer A supplemented with 10 mM imidazole. Using a step gradient, the column was first washed with 10 column volumes of buffer A supplemented with 100 mM imidazole and the prenyltransferases were eluted with 5 column volumes of buffer A supplemented with 500 mM imidazole. The resulting enzyme preparations were dialyzed against 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 10% glycerol, twice. After dialysis, glycerol was added to bring the solution to 50%, and then the solution was frozen at -80°C.

Site-Directed Mutagenesis of *ispA* and In Vitro Prenyltransferase Assay

Wild-type *ispA* was isolated from the genomic DNA of a single *E. coli* colony by PCR using the Roche HF-PCR system and TA-cloned into pCR4 (Invitrogen) resulting in the plasmid pCRispA. PCR-mediated site-directed mutations were carried out using the Quick-Change kit (Stratagene) and the primers S81F-for, S81F-rev, Y80D-for, and Y80D-rev (Table II) with the plasmid pCRispA as a template. The mutations were confirmed by DNA sequencing.

The *E. coli* wild-type and mutant prenyltransferases were assayed in vitro to determine their product profiles and enzymatic activity levels. Five microliters of purified protein were diluted into 45 μL of warmed reaction buffer (50 mM Tris-HCL pH 7.5, 30 mM $MgCl_2$, 37°C) to final

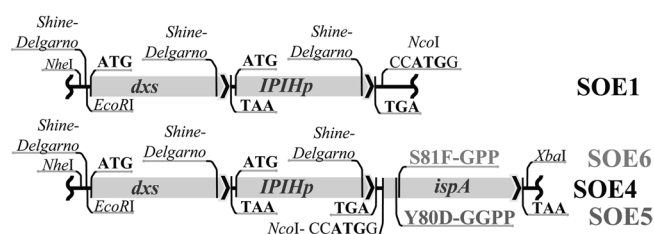


Figure 2. Diagram of the engineered SOE operons. For each gene the start and stop codons are indicated along with the position of introduced restriction and Shine-Dalgarno sites.

substrate and enzyme concentrations as follows: 400 μ M of IPP and DMAPP to 6.7 μ M IspA (Ser81Phe), 540 μ M of IPP and 270 μ M of DMAPP to 1.2 μ M IspA (WT), and 600 μ M of IPP and 200 μ M of DMAPP to 6.5 μ M IspA (Tyr80Asp). The reaction proceeded at 37°C overnight. To quench the reaction and increase the sensitivity of HPLC analysis, 10 μ L of 100 mM EDTA was added to the reaction. Forty microliters of the quenched reaction were loaded onto a C₁₈ reverse-phase high-performance liquid chromatography (HPLC) column previously equilibrated with buffer I (25 mM NH₃HCO₂). Buffer I was run for an additional 30 s after sample loading followed first by a 4-min gradient from 100% buffer I to 100% buffer II (100% acetonitrile) and finally by a 1-min wash with buffer II. A standard for each polyprenyl diphosphate (FPP, GPP, GGPP) was run using this HPLC protocol at three different concentrations to derive the relationship between HPLC peak area and polyprenyl diphosphate concentration.

In Vivo Lycopene Assay

To analyze the flux to GGPP, we utilized a previously described assay for lycopene production (Cunningham et al., 1994; Kim and Keasling, 2001). Briefly, the plasmid pAC-LYC consists of the genes encoding GGPP synthase, phytoene synthase, and lycopene synthase genes from *Erwinia herbicola* (Fig. 1). DH5 α cells, cotransformed with either the FPP or the GGPP biosynthetic operon and the constitutive lycopene operon, were inoculated into 2YT medium and grown to an OD₆₀₀ of 2.5. To induce gene expression, aliquots of cultures were diluted to a final OD₆₀₀ of 0.1 into fresh 2YT medium containing arabinose. These cultures were shaken in a 29°C water bath for 24 h, at which time cells from 1 mL of culture medium were harvested by centrifugation for 2 min at 18,188 RCF and room temperature. The supernatant was discarded and 1 mL of acetone was added, followed by incubation in a 55°C-water bath for 5 min. The acetone-extracted lycopene from each sample was assayed by measurement of the absorbance at 470 nm.

Expression of Diterpene Cyclases and GC-MS Analysis of Products

Diterpene production was confirmed for each cyclase using gas chromatography-mass spectrometry (GC-MS) analysis of ethyl acetate-extracted cultures. For the diterpene cyclases, overnight cultures were grown from a single colony and used to inoculate (1% v/v) 50 mL of medium. The cultures were grown at 37°C until an OD₆₀₀ of 0.4 at which time they were induced with 1 mM IPTG and 13 mM arabinose. Cells were harvested after 4 h of post-induction growth, and pellets suspended in 1 mL of phosphate-buffered saline. For full-scan GC-MS analysis, diterpenes from 0.7 mL of suspended cells were extracted with an equal volume of ethyl acetate. Extractions were carried out in glass vials to avoid contaminants that may be extracted

from plastic tubes. Initial studies on diterpenes explored the products of a pBBRSOE5/pTrcKau *E. coli* in a DH5 α background. To minimize background and maximize signal, these studies were performed in M9 medium and the prepared ethyl acetate extracts were concentrated up to 20-fold by a gentle flow of nitrogen gas prior to GC-MS analysis. For later studies with casbene, the greater signal due to the activity of the cyclase and the more robust growth of *E. coli* in LB warranted a switch to this media. To identify the diterpenes, total ion scans were run on the GC-MS monitoring the 40 to 275 (m/z) ions. For time course analysis with pBBRSOE5/pTrcCas, 0.7 mL of growing culture was extracted with an equal part of ethyl acetate. To increase the sensitivity and selectivity of detection, the MS was operated in selected ion-monitoring (SIM) mode using ions of 121, 136, 257, and 272 m/z, which represent the molecular ion and three abundant ions of casbene. The diterpene cembrene was used as an internal standard for chromatographic runs and to calibrate casbene production. All the diterpene GC-MS analyses utilized a Hewlett Packard HP6890 gas chromatograph equipped with a Hewlett Packard 5973 mass selective detector and a HP-5MS capillary column (30M $\times$ 250 μ m ID $\times$ 0.25 μ m film thickness). Diterpenes from splitless 1- μ L injections were separated using a GC oven temperature program of 80°C for 2 min, followed by a 10°C/min ramp to 300°C. Injector and MS quadrupole detector temperatures were 250°C and 150°C, respectively.

In Vivo and In Vitro GC-MS Analysis of Monoterpene Cyclase Products

For detection of monoterpenes, a solid-phase microextraction (SPME; Supelco) filament was exposed to the headspace of samples. Each 100- μ m polydimethylsiloxane filament was conditioned before use by exposing it to the 250°C injection chamber for 2 h with the split set to divert flow away from the column. A filament was considered ready for use when two consecutive blank GC-MS runs exhibited overlapping baseline traces. Each filament was used for less than 100 samplings with blanks run between experiments to confirm the integrity of the filament. For headspace sampling, terpenes were eluted from the impregnated filament by a 2-min exposure to the 220°C GC injection chamber. For the analysis of monoterpenes, the GC-MS was equipped with a cyclo-sil B chiral column (30M $\times$ 250 μ m id $\times$ 0.25 μ m film thickness, Agilent Technologies). The GC oven program used for all SPME runs was as follows: a column loading at 60°C for 4 minutes, followed by a ramp at 10°C/minute to 140°C and a second ramp at 80°C/minute to 200°C where the temperature was held for 2 min. For full scans, an ion range of 40 to 140 (m/z) was monitored. In SIM mode, only the 136, 121, and 93 ions were monitored.

For in vitro monoterpene production, overnight cultures of a pBBRSOE6/pTrcCar cotransformed strain were grown from a single colony and used to inoculate (1% v/v) three

30-mL cultures of LB medium. Cultures were grown to an OD₆₀₀ of 0.4, induced with 1 mM IPTG and 0.13 mM arabinose, and grown for an additional 6 h in 250-mL, baffled, shake flasks. These cultures were pelleted and frozen at -80°C . Cells from frozen pellets were suspended in 1 mL of assay buffer (25 mM Hepes pH 7.2, 100 mM KCl, 10 mM MnCl₂, 10% glycerol, and 5 mM DTT) and lysed on ice by sonication via three iterations at 50% duty cycle for 1 min. Lysates were clarified by centrifugation at 17,530 RCF for 20 min at 4°C . A 100- μL aliquot of the supernatant was diluted into 900 μL of assay buffer in a 4.5-mL serum vial. A one- μL aliquot of 1 mg/mL solution of GPP in water was added to the serum vial bringing the substrate concentration to 2 μM GPP, and then the vial was sealed. The reactions were allowed to proceed for 30 min at room temperature. The SPME filament was exposed to the headspace of the in vitro reactions for 30 s. The adsorbed volatiles were desorbed directly into the 220°C GC-MS injection port for 2 min.

For in vivo monoterpene production, overnight cultures cotransformed with pTrcCar and pBBRSOE6 were grown from a single colony and used to inoculate (1% v/v) three 200-mL cultures of LB medium in baffled, 500-mL flasks. The start of each culture was staggered by 20 min to allow time for SPME sampling between replicate measurements. Cultures were grown to an OD₆₀₀ of 0.4, induced with 1 mM IPTG and 0.13 mM arabinose and shaken for an additional 5 min to ensure thorough mixing of the inducer. Thirty-mL aliquots of the induced culture were placed into 250-mL serum vials and capped with Teflon septa. A new vial was used for each time point measurement. In sampling, each Teflon septum was pre-puncture with a 21-gauge needle taking care to cover the end of the needle to prevent outgassing from the culture. The SPME filament was exposed to the headspace of a serum vial in a 32°C water bath for 8 min. As the interval between removing the filament from the headspace and inserting the SPME into the GC-MS affected the total yields, the sampling set-up and the GC-MS equipment were colocalized. Calibration curves for 3-carene were determined using an 8-min exposure of the SPME filament to the headspace of LB medium warmed to 32°C and spiked with each standard (Fluka) to a final concentration of 1, 10, 100, and 1000 $\mu\text{g/L}$.

RESULTS

Conversion of the *E. coli* FPP Cyclase (IspA) to Either a GGPP or GPP Synthase

Preliminary experiments showed that the synthase from *Archaeoglobus fulgidus* (Gps) performed poorly when ex-

pressed in *E. coli* (data not shown). We suspected that poor expression of the *gps* gene was due to an abundance of rare Arg codon. To circumvent this and other potential pro-

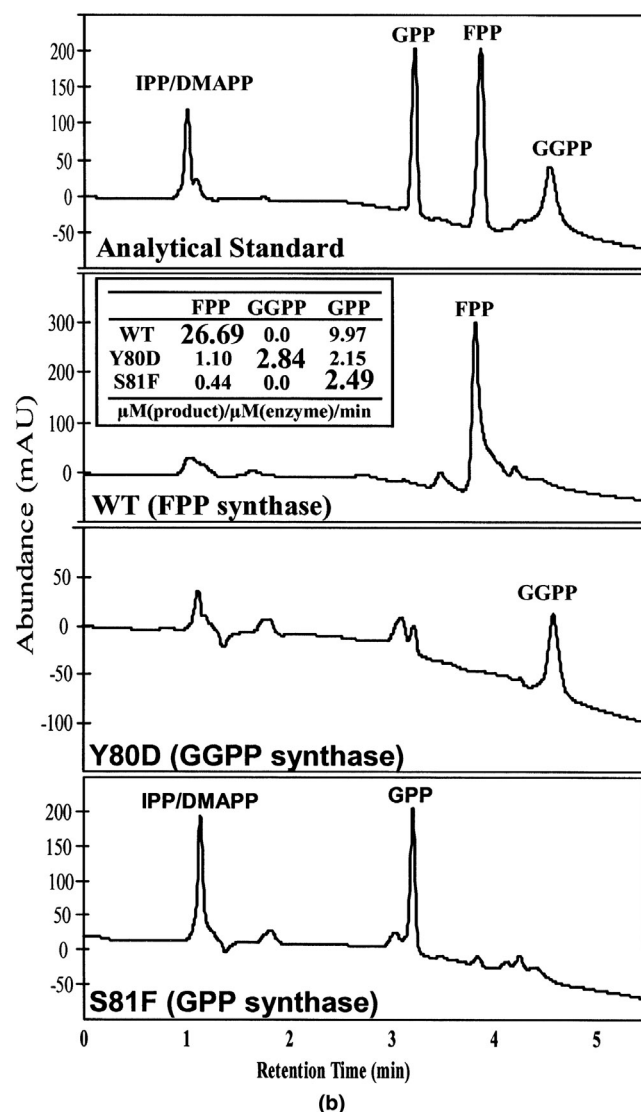
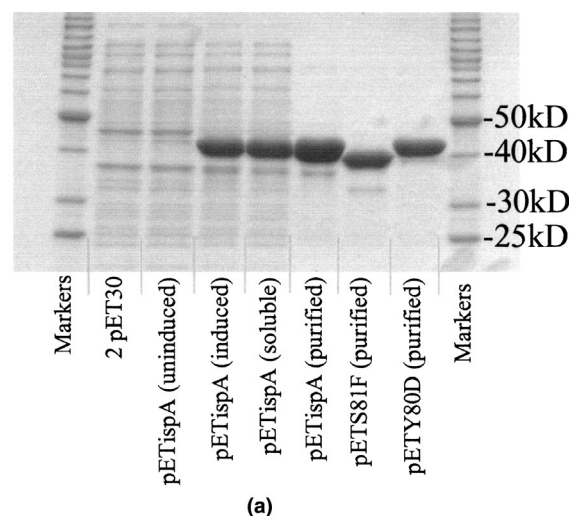


Figure 3. Purification and characterization of the polyprenyl diphosphate synthase (IspA) variants. IspA wild-type and Ser81Phe and Phe80Asp variants were purified and their product profiles characterized. (a) SDS-PAGE gel loaded with the samples as marked and indicating the progression to purified enzymes. (b) Stacked HPLC traces of the IspA variant activity assays along with the analytical standards.

blems associated with heterologous protein expression, a GGPP synthase and a GPP synthase were constructed by introducing mutations into the active site of the native *E. coli* FPP synthase gene, *ispA*. Based on previous studies, which demonstrated that GPP or GGPP could be produced by a mutant form of FPP synthase (Ohnuma et al., 1996a), two amino acids in the *E. coli* FPP synthase (IspA) were targeted for mutation. For the first mutation, Tyr80 of IspA was mutated to an Asp residue producing a putative GGPP synthase. For the second mutation, the codon specifying Ser81 of IspA was changed to a Phe codon to produce a putative GPP synthase.

Before in vivo expression studies, the shift in product specificity for the two mutant prenyltransferases was confirmed in vitro with purified enzymes. The purified his-tagged wild-type, Tyr80Asp, and Ser81Phe IspA variant enzyme preparations were more than 95% pure, as assayed by Coomassie-stained SDS-PAGE protein gel electrophoresis (Fig. 3a). Purified synthases were provided IPP and DMAPP as substrates and the products were analyzed using HPLC-UV. The comparison of the retention times of the prenyl diphosphates produced by the enzymes to those of analytical standards confirmed the production of GGPP and GPP by the Tyr80Asp and Ser81Phe variant, respectively (Fig. 3b). Although detailed enzyme kinetics studies were not performed, in vitro analysis confirmed that these mutations in IspA had the predicted effect in changing product specificity. When the enzymatic reactions were allowed to go to completion, the Tyr80Asp and Ser81Phe formed GGPP and GPP as in excess of 80% and 90% of the final product mixture, respectively. As might be expected from the results of the *Bacillus*-engineered synthases, the relative reaction rates of the engineered synthases compared to the wild-type enzyme also showed a reduction in overall activity. Although the product specificities of the IspA enzymes may have been influenced by the substrate

concentrations and ratios and by the incubation time, these results demonstrate that the product distribution shifts in the mutant enzymes in the predicted fashion.

Engineered Isoprenoid Biosynthetic Operons Increase Lycopene Production In Vivo

To augment the intracellular pools of GPP and GGPP in *E. coli*, we constructed three operons containing combinations of the following genes: DXP synthase (*dxs*), IPP isomerase (*IPPHp*), and wild-type or variants of the *E. coli* FPP synthase (*ispA*). The production of GGPP and FPP was measured using lycopene biosynthesis as a reporter. The arabinose-induced lycopene production by cells containing the SOE4(FPP) operon was sixfold higher than that by cells without any metabolic augmentation (*E. coli* strain DH5 α) (Fig. 4) indicating that the SOE4 operon increased flux through the isoprenoid biosynthetic pathway. To assess the biosynthesis of GGPP in vivo, lycopene production was measured in strains cotransformed with pBBRSOE5 and pACYC-IB. The pACYC-IB plasmid differs from the previously described pAC-LYC plasmid by the deletion of the *crtE* gene, which encodes an FPP to GGPP synthase (Ohnuma et al., 1994). Therefore, lycopene production by this strain was dependent almost entirely upon the capacity of the SOE5 operon to produce GGPP. In vitro studies on the *E. coli* enzyme IspB indicate that some GGPP may be present in the cell as an intermediate in the production of octaprenyl diphosphate and thus may support a minimal lycopene background (Asai et al., 1994; Kainou et al., 2001). Arabinose-inducible lycopene production was observed for the strains cotransformed with pBBRSOE5 and pACYC-IB in contrast to an absence of lycopene in the control strains cotransformed with pBBRSOE4 and pACYC-IB (Fig. 4). The peak levels of lycopene production achieved in with the

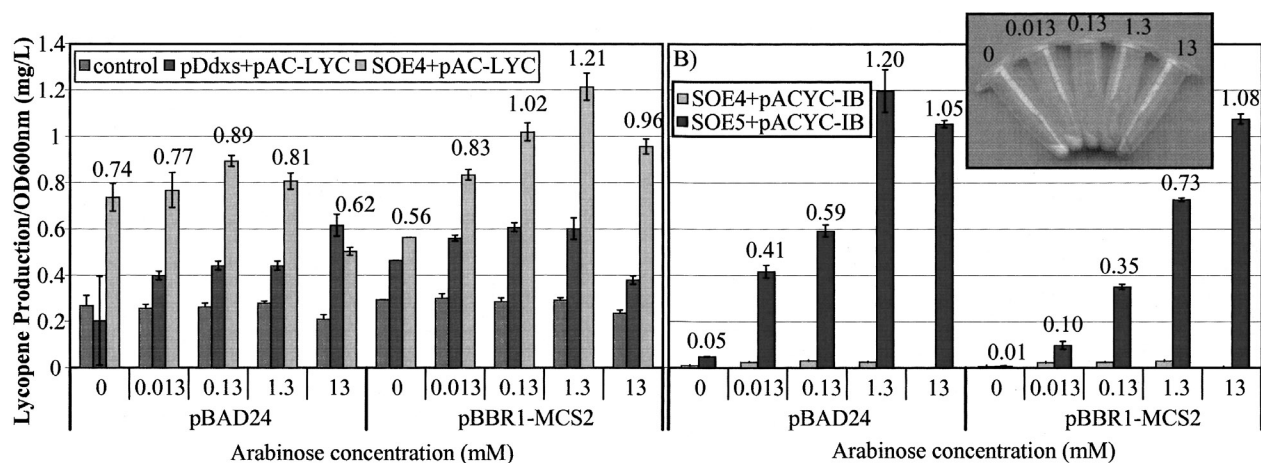


Figure 4. Lycopene production using the SOE4(FPP) and SOE5(GGPP) operons in the vectors pBAD24 (A) or pBBR1-MCS2 (B) at various arabinose concentrations. The insert is a picture of concentrated arabinose-induced, lycopene-producing cells, which exhibit the characteristic red pigmentation of lycopene.

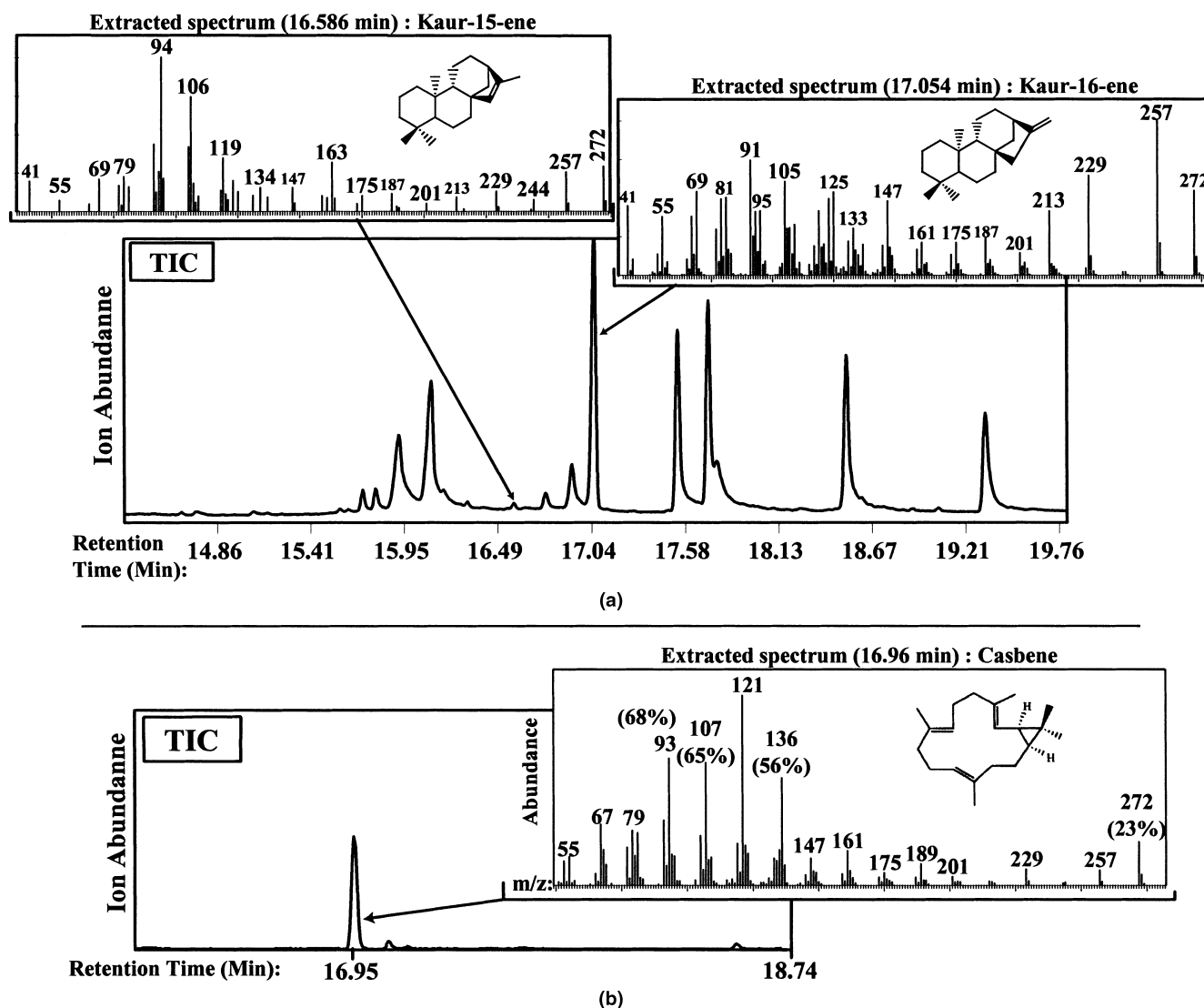


Figure 5. GC-MS detection of in vivo diterpene production. (a) GC-MS trace and full scan mass spectra of an ethyl acetate extraction of an *E. coli* strain cotransformed with pTrcKau and pBBRSOE5(GGPP). (b) GC-MS trace and mass spectra as in (a) but with casbene cyclase.

pBBRSOE4 and pAC-LYC (1.21 ± 0.03 mg/L) were similar to those strains cotransformed with pBBRSOE5 and pACYC-IB (1.08 ± 0.02 mg/L) indicating that in vivo the native IspA is not limiting in lycopene production and that GGPP synthesis by Tyr80Asp is no more limiting than CrtE. Since two molecules of GGPP are required to make lycopene, this enhanced capacity should result in twice the level of diterpenes as lycopene, assuming no other limitations such as a poor expression or activity of the diterpene cyclases.

Diterpene Production in *E. coli*

To produce mono- and diterpenes in vivo using prenyl diphosphate precursors produced by the isoprenoid operons, a diterpene or monoterpene cyclase was coexpressed in *E. coli* with either SOE5(GGPP) or SOE6(GPP). A pTrc99 a-derived plasmid expressing either the casbene cyclase

(pTrcCas) or *ent*-kaurene cyclase (pTrcKau) was cotransformed with pBBRSOE5, and the resulting *E. coli* strains were assayed for diterpene production by GC-MS (Fig. 5).

Lacking commercially available standards, confirmation of casbene and kaurene production was based on comparison of experimental spectra with published spectra for these compounds (Fig. 5). To ensure the compound analyzed was a product of the cyclase, the spectra from cells harboring the pBBRSOE5/pTrc(cyclase) were compared to the spectra of the control strain (pBBRSOE5/pTrc99 a). The relative ion abundances for the bacterially produced casbene exhibited a 97% correlation coefficient with the published values for the 121, 93, 107, 136, and 272 m/z ions (peak heights normalized to most abundant ion): 1, 0.92, 0.71, 0.54, and 0.14 vs. 1, 0.75, 0.68, 0.61, and 0.26 for published (Guilford and Coates 1982) and experimental spectra, respectively (Fig. 5b). *Ent*-kaurene cyclase produced two diterpenes, kaur-16-ene and kaur-15-

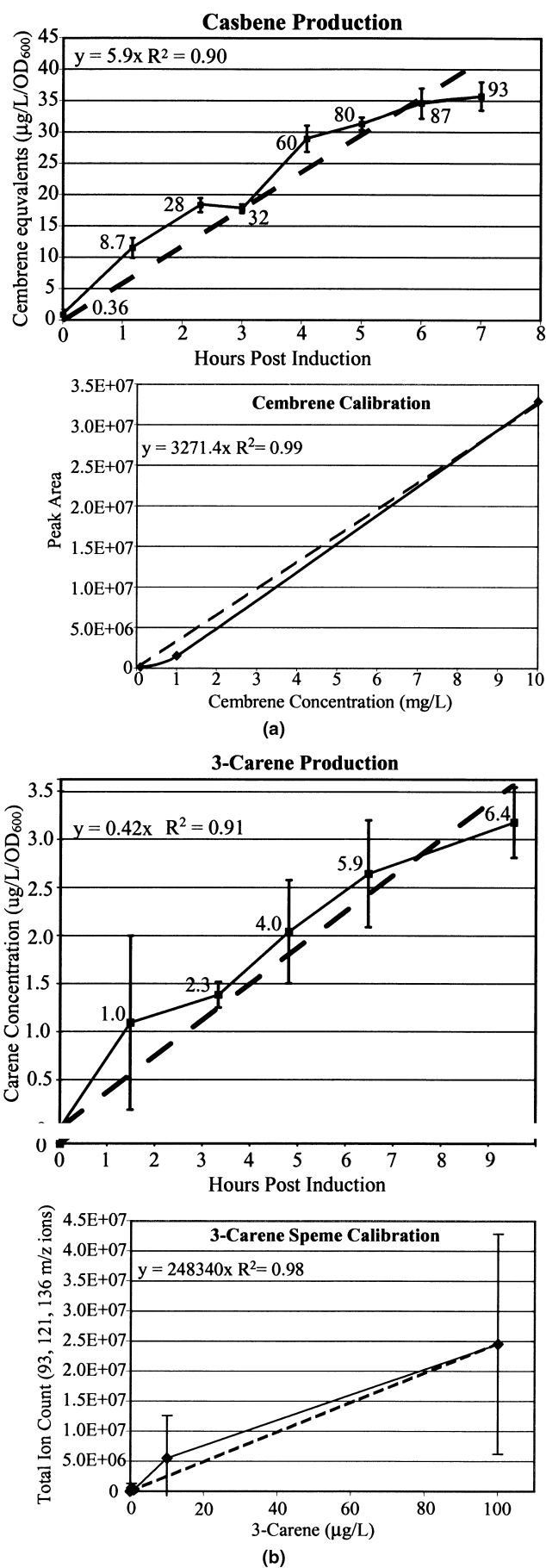
ene, with relative abundances of 100% and 6.7%, respectively. The extracted spectra for the two products exhibited 99% and 97% matches to library spectra of kaur-16-ene and kaur-15-ene, respectively (Fig. 5a).

Casbene cyclase was selected for further characterization of its production via time-course experiments. Growth inhibition occurred at arabinose levels above 0.13 mM (data not shown); therefore, 0.13 mM was chosen for all production runs. Casbene cyclase was able to produce peak casbene titers of roughly 30 $\mu\text{g}/\text{OD}_{600}/\text{L}$ with production peaking at 7 h (Fig. 6a). The specific casbene production rate was roughly 6 $\mu\text{g}/\text{OD}_{600}/\text{L}/\text{h}$ with a goodness of fit (R^2) of 90%. Huang et al. (2001) produced taxadiene in vivo at final titers of 1.3 mg/L after a 24-h incubation. Based on our measured production rate and final OD_{600} of 2.5, we might expect casbene titers of 0.3 mg/L. The cells producing casbene grew as well as the control cells, reaching an average OD_{600} of 2.4 vs. 2.5 for control cultures. From these data, we conclude that there is no appreciable toxicity to the host producing casbene at these levels.

Monoterpene Production in *E. coli*

The monoterpene 3-carene cyclase was cotransformed with pBBRSOE6 and the cultures assayed for monoterpene production. 3-Carene, like most monoterpenes, is quite volatile and thus necessitated a sampling technique different from that used for diterpenes. Headspace analysis of pBBRSOE6/pTrcCar cotransformed cultures was performed using SPME. 3-Carene cyclase was reported previously to have multiple side products in addition to the major 3-carene product (Faldt et al., 2003). Unfortunately, the in vivo production level of the minor components of the SIM trace was not sufficient to collect full ion scans for compound identification. Therefore, GC-MS full scans were performed on headspace samples of in vitro cyclase activity assays run using crude cell lysates. Total peaks' areas of 460 million counts were seen for the in vitro traces allowing the subsequent identification of the following products for 3-carene cyclase: α -pinene (0.9%), myrcene (4.6%), sabinene (5.9%), 3-carene (100%), γ -terpinene

Figure 6. Di- and monoterpene production curves. For both graphs, the primary axis is reported in units of production per liter per cell density to better reflect the productivity of the bacteria. Next to each point is listed the per liter production to aid in comparison to past studies and indicate the total productivity of the cultures. (a) An 8-h casbene production run sampled hourly in triplicate. The Y-axis is in cembrene, a related diterpene, equivalents as determined by scaling relative abundances of the 121, 93, and 272 ions from the casbene run to standard curves of cembrene. Included is the calibration graph used to relate casbene peak height to cembrene concentration or casbene equivalents: 10, 1, and 0.1 mg/L cembrene. (b) A 9-hour 3-carene production run sampled hourly in triplicate using SPME headspace analysis. The Y-axis is 3-carene production scaled to a standard curve. The calibration curve relating a mass spectra peak area to a concentration of 3-carene standard is shown. 3-Carene concentrations of 0.01, 0.1, 0.33, 1, 10, and 100 $\mu\text{g}/\text{L}$ were used in the calibration experiment.



(1.1%), limonene (0.8%), β -phellandrene (1.9%), α -terpinene (2.0%), and terpinolene (15.5%) (Fig. 7). For α -pinene, myrcene, 3-carene, γ -terpinene, α -terpinene, and terpinolene, the identity of each peak was corroborated by co-elution with an analytical standard.

In vivo production curves for pBBRSOE6/pTrcCar co-transformed strains were obtained by monitoring the monoterpene GC-MS peaks identified in the above in vitro experiments. Based on these data, 3-carene cyclase was able to produce a peak 3-carene titer of $\approx 3 \mu\text{g/L/OD}_{600}$ after 8 h

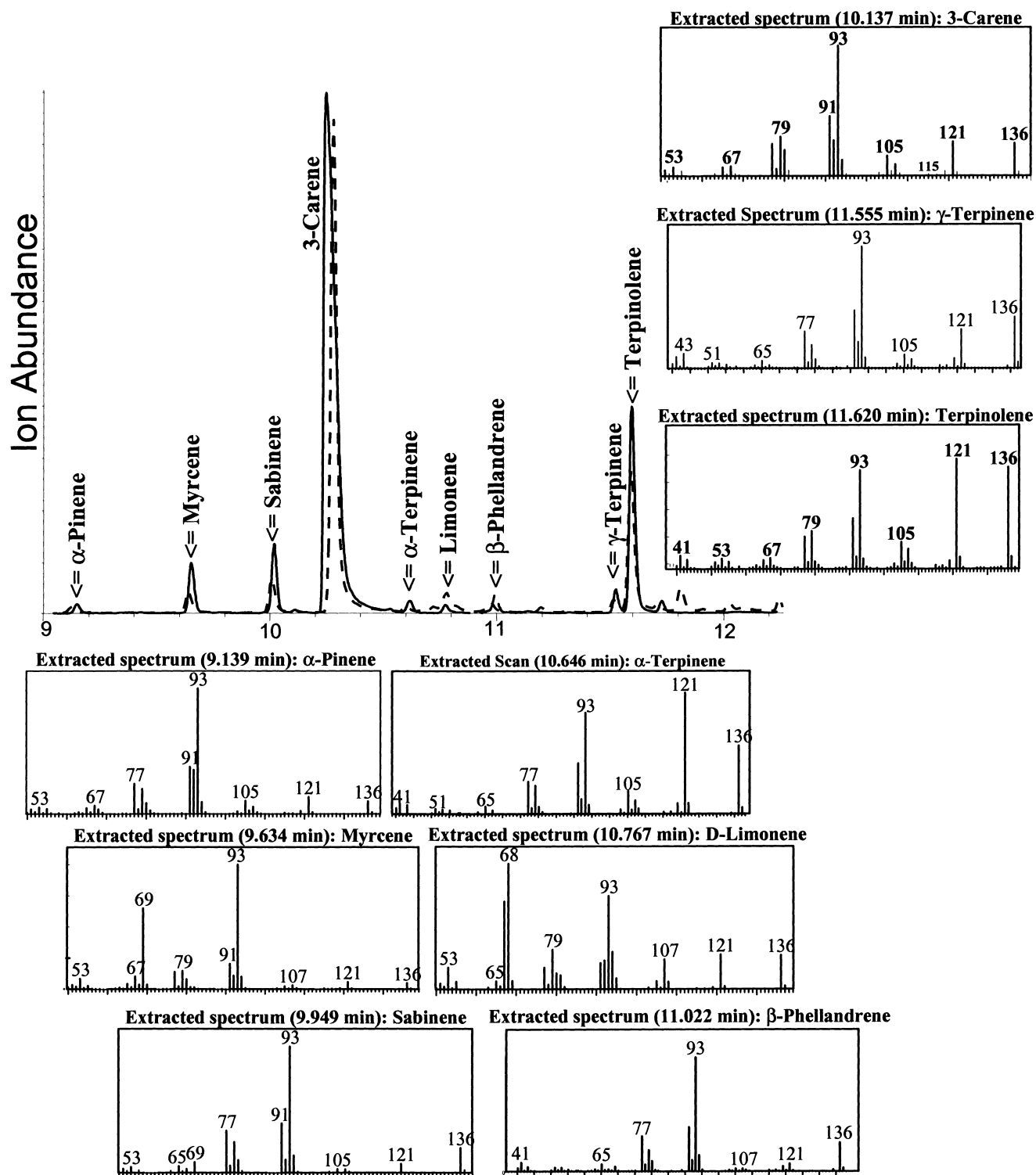


Figure 7. GC-MS chromatography traces and mass spectrum for in vitro and in vivo carene cyclase. In vitro (solid) and in vivo (dashed) GC-MS traces are overlaid with a scale factor of 1000 applied to the in vivo data. Arrayed around the trace are full mass spectra for the nine identified monoterpenes.

of production (Fig. 6B). Fitting this production curve to a line, the specific carene production rate was roughly $0.42 \mu\text{g}/\text{OD}_{600}/\text{L}/\text{h}$ with a goodness of fit (R^2) to a line of 91%.

DISCUSSION

To increase the intracellular pools of the polyprenyl diphosphates GPP and GGPP, we have augmented components of the polyprenyl diphosphate biosynthetic pathway in *E. coli*. Multiple labs, to include ourselves, have previously demonstrated that overproduction of the IPP biosynthesis enzymes Dxs and Dxr increase the flux to the intracellular pool of FPP (Harker and Bramley, 1999; Kim and Keasling, 2001; Matthews and Wurtzel, 2000; Wang et al., 1999). These studies demonstrated that *dxs* overexpression has a greater impact on lycopene yields than does overexpression of *dxr* (Kim and Keasling, 2001). In addition, several studies reported enhanced carotenoid synthesis from the overexpression of an isopentenyl diphosphate isomerase, such as IPPHp, or a prenyl diphosphate synthase, such as CrtE or Gps (Albrecht et al., 1999; Kajiwarra et al., 1997; Sun et al., 1998; Wang et al., 1999). Our constructed isoprenoid SOE operons drive flux through the native isoprenoid pathway in *E. coli* to the polyprenyl diphosphates, GPP, FPP, and GGPP.

Because of the vagaries of foreign protein production in *E. coli* (codon usage and quaternary or tertiary protein structure), we chose to derive a GGPP or GPP synthase from the native *E. coli* FPP synthase IspA. In prokaryotic prenyltransferases, the product specificity of the enzyme is determined by the presence of an aromatic acid residue located in the active site pocket of the enzyme (Wang and Ohnuma, 2000). Using the *Bacillus stearothermophilus* FPP synthase, Ohnuma et al. (1996a) demonstrated that mutation of the tyrosine, five residues preceding the first of two conserved aspartate-rich motifs (FARM), to a non-aromatic amino acid switches the product specificity from FPP to GGPP. In an analogous study, Narita et al. (1999) generated a GPP synthase by a mutation four residues preceding the FARM. Using these studies as a guide, we generated a GPP and a GGPP synthase from the native *E. coli* FPP synthase IspA. The altered production profiles of these engineered IspA variants were confirmed in vitro using purified proteins (Fig. 3b).

We used lycopene production as an assay to evaluate the isoprenoid flux in *E. coli* and to compare the SOE4(FPP) and SOE5(GGPP) operons (Fig. 4). The SOE5(GGPP) operon achieved peak lycopene production of $1.08 \text{ mg}/\text{L}/\text{OD}_{600}$, with sixfold higher yields of lycopene than was seen with the wild-type strain. Although direct lycopene yield comparison to previous studies is difficult because of differences in the strains and assay conditions used, our results agree with published values of 2- to 50-fold increases in carotenoid yields and a synergistic effect when overexpressing pairs of *dxs*, *IPPHp*, and *ispA* (Albrecht et al., 1999; Kajiwarra et al., 1997; Sun et al., 1998; Wang et al., 1999).

The in vitro IspA assays reported here demonstrate that the IspA(Tyr80Asp) variant contained in the SOE5(GGPP) operon is indeed a GGPP synthase. This engineered IspA has a lower enzymatic activity than that of the wild-type IspA enzyme, the gene is expressed in the SOE4(FPP) operon. The ratio of pBBRSOE5(GGPP) lycopene production to pBBRSOE4(FPP) lycopene production ranged from 0.15–1.37 when comparing identical arabinose concentrations. Thus, the in vivo activity of IspA(Tyr80Asp) may exceed that measured by the in vitro prenyltransferase assay, or the reduced activity of the mutant enzyme may simply outpace precursor or lycopene production. Addressing the first alternative, heterodimeric prenyltransferase formed from the intracellular expression of the wild-type and mutant prenyltransferases may show different activity (Koyama et al., 2000) or the lower intracellular substrate concentrations may modify chain length specificity (Ohnuma et al., 1997). As a caveat to the above discussion, each operon, at full induction, reached a maximum lycopene production of approximately $1.1 \text{ mg}/\text{OD}_{600}/\text{L}$. This common productivity may represent the ceiling on lycopene production due to the reporter assay or *E. coli* physiology and thus would prevent the evaluation of FPP and GGPP production from the operons outside this range. Despite a reduction in activity, the mutant IspA(Tyr80Asp) of the SOE5(GGPP) operon does not appear to further limit GGPP production and thus flux to a terpene cyclase.

Mono and Diterpene Production in *E. coli*

We next looked at mono and diterpene production supported by the low copy pBBRSOE(5/6) operons. For monoterpene production, pBBRSOE6/pTrcCar strains produced 10-fold less terpene than did the pBBRSOE5/pTrcCas strains: 3 vs. $30 \mu\text{g L OD}_{600}$. In vitro studies demonstrated that the 3-carene cyclase was quite active and capable of producing large quantities of monoterpenes. Carter et al. (2003) recently demonstrated the production of limonene in *E. coli*. They demonstrated the synergistic coupling of a GPP synthase and an IPP isomerase in the production of a monoterpene and achieved production levels of $5 \text{ mg}/\text{L}$ in 24-h culture runs. It is difficult to compare these results to ours as they utilized quite different growth and production conditions and strains. They, however, did not extend their engineering efforts to the modification of the DXP pathway, which our results suggest would have benefited their system. Comparison of our monoterpene and diterpene results suggests that less GPP is available from the SOE6 operon than GGPP from the SOE5 operon. Scavenging of GPP for FPP production by both the endogenous and the Ser81Phe variant of IspA may reduce intracellular GPP pools. Further, the native IspA, being more active than the engineered IspA, may outpace GPP production.

Previous investigations indicate poor protein production in *E. coli* for both diterpene cyclases used in the study (Hill et al., 1996; Huang et al., 1998; Kawaide et al., 2000).

While protein production was not rigorously quantified in this study, neither diterpene cyclase was visible by SDS-PAGE analysis upon induction. Despite this, we were able to detect *in vivo* cyclase activity of both enzymes and identify, for the first time, the minor kaur-15-ene side-product of the *ent*-kaurene cyclase.

High-level production of polyprenyl diphosphates may prove useful in the production of a wide variety of compounds such as plant hormones (gibberellins), antibacterial/fungal agents (phytoalexins), and anticancer drugs (Taxol). The engineering of prenyltransferases with high activity and specificity is the first step toward the biosynthesis of the terpene precursors GPP and GGPP in *E. coli*. However, lower monoterpene production by the recombinant carene synthase compared to the casbene cyclase may indicate that in *E. coli*, GPP is consumed by the native FPP synthase. Higher yields of monoterpenes should be obtained by a combination of increasing monoterpene cyclase activity and flux to GPP. Our recent study showed that the use of heterologous pathways can provide an alternate method of increasing isoprenoid flux (Martin et al., 2003). While mono and diterpene productions levels reported here are unsuitable for commercialization, the results of this study combined with our previously engineered IPP-producing platform strain should facilitate the *in vivo* screening and production of mono and diterpene cyclases in *E. coli*. Furthermore, these combined results set the stage for the discovery of terpene modifying enzymes such as cytochrome P450s.

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References

- Albrecht M, Misawa N, Sandmann G. 1999. Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β -carotene and zeaxanthin. *Biotechnol Lett* 21:791–795.
- Apfel CM, Takacs B, Fountoulakis M, Stieger M, Kekk W. 1999. Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: Cloning, expression, and characterization of the essential *uppS* gene. *J Bacteriol* 181(2):483–492.
- Asai K, Fujisaki S, Nishimura Y, Nishino T, Okada K, Nakagawa T, Kawamukai M, Matsuda H. 1994. The identification of *Escherichia coli* *ispB* (*cel*) gene encoding the octaprenyl diphosphate synthase. *Biochem Biophys Res Commun* 202(1):340–345.
- Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD et al. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* 277(5331):1453–1474.
- Boucher Y, Doolittle WF. 2000. The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. *Mol Microbiol* 37(4):703–716.
- Carter OA, Peters RJ, Croteau R. 2003. Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry* 64(2):425–433.
- Cunningham FX Jr, Sun Z, Chamovitz D, Hirschberg J, Gantt E. 1994. Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. *Plant Cell* 6(8):1107–1121.
- Erkel G, Anke T. 1994. Hyphodontal, a new antifungal inhibitor of reverse transcriptases from *Hyphodontia* sp. (Corticaceae, Basidiomycetes). *Z Naturforsch* 49c:561–570.
- Faldt J, Martin D, Miller B, Rawat S, Bohlmann J. 2003. Traumatic resin defense in Norway spruce (*Picea abies*): Methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol Biol* 51(1):119–133.
- Ferrandiz ML, Sanz MJ, Bustos G, Paya M, Alcaraz MJ, De Rosa S. 1994. Avarol and avarone, two new anti-inflammatory agents of marine origin. *Eur J Pharm* 253:75–82.
- Fraga BM. 1991. Sesquiterpenoids. In: Charlwood BV, Banthorpe DV, editors. *Methods in plant biochemistry*. San Diego, CA: Academic Press. p 145–185.
- Gelb MH, Tamanoi F, Yokoyama K, Ghomashchi F, Esson K, Gould MN. 1995. The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. *Cancer Lett* 91(2):169–175.
- Gould MN. 1997. Cancer chemoprevention and therapy by monoterpenes. *Environ Health Perspect* 105(S4):977–999.
- Guilford WJ, Coates RM. 1982. Stereochemistry of casbene biosynthesis. *J Am Chem Soc* 104:3506–3508.
- Harker M, Bramley PM. 1999. Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatase in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett* 448(1):115–119.
- Hill AM, Cane DE, Mau CJ, West CA. 1996. High level expression of *Ricinus communis* casbene synthase in *Escherichia coli* and characterization of the recombinant enzyme. *Arch Biochem Biophys* 336(2):283–289.
- Hohl RJ. 1996. Monoterpenes as regulators of malignant cell proliferation. In: Research AICR, editor. *Dietary phytochemicals in cancer prevention and treatment*. New York: Plenum Press.
- Huang K, Huang Q, Scott AI. 1998. Overexpression, single-step purification, and site-directed mutagenetic analysis of casbene synthase. *Arch Biochem Biophys* 352(1):144–152.
- Huang Q, Roessner CA, Croteau R, Scott AI. 2001. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorg Med Chem* 9(9):2237–2242.
- Jennewein S, Croteau R. 2001. Taxol: Biosynthesis, molecular genetics, and biotechnological applications. *Appl Microbiol Biotechnol* 57(1–2):13–19.
- Kainou T, Okada K, Suzuki K, Nakagawa T, Matsuda H, Kawamukai M. 2001. Dimer formation of octaprenyl-diphosphate synthase (*IspB*) is essential for chain length determination of ubiquinone. *J Biol Chem* 276(11):7876–7883.
- Kajiwara S, Fraser PD, Kondo K, Misawa N. 1997. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem J* 324(Pt 2):421–426.
- Kawaide H, Imai R, Sassa T, Kamiya Y. 1997. *Ent*-kaurene synthase from the fungus *Phaeosphaeria* sp. L487. cDNA isolation, characterization, and bacterial expression of a bifunctional diterpene cyclase in fungal gibberellin biosynthesis. *J Biol Chem* 272(35):21706–21712.
- Kawaide H, Sassa T, Kamiya Y. 2000. Functional analysis of the two interacting cyclase domains in *ent*-kaurene synthase from the fungus *Phaeosphaeria* sp. L487 and a comparison with cyclases from higher plants. *J Biol Chem* 275(4):2276–2280.
- Kim SW, Keasling JD. 2001. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol Bioeng* 72(4):408–415.
- Koyama T, Gotoh Y, Nishino T. 2000. Intersubunit location of the active site of farnesyl diphosphate synthase: Reconstruction of active enzymes by hybrid-type heteromeric dimers of site-directed mutants. *Biochemistry* 39(2):463–469.
- Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. 2003. Engineering the mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol* 21:796–802.
- Matthews PD, Wurtzel ET. 2000. 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(4):396–400.
- Mau CJ, West CA. 1994. Cloning of casbene synthase cDNA: Evidence for conserved structural features among terpenoid cyclases in plants. *Proc Natl Acad Sci USA* 91(18):8497–8501.
- McCaskill D, Croteau R. 1997. Prospects for the bioengineering of isoprenoid biosynthesis. *Adv Biochem Eng/Biotechnol* 55:107–146.
- McGarvey DJ, Croteau R. 1995. Terpenoid metabolism. *Plant Cell* 7: 1015–1026.
- Narita K, Ohnuma S, Nishino T. 1999. Protein design of geranyl diphosphate synthase. Structural features that define the product specificities of prenyltransferases. *J Biochem* 126(3):566–571.
- Ohnuma SI, Hirooka K, Ohto C, Nishino T. 1997. Conversion from archaeal geranylgeranyl diphosphate synthase to farnesyl diphosphate synthase: Two amino acids before the first aspartate-rich motif solely determine eukaryotic farnesyl diphosphate synthase activity. *J Biol Chem* 272(8):5192–5198.
- Ohnuma SI, Narita K, Nakazawa T, Ishida C, Takeuchi Y, Ohto C, Nishino T. 1996a. A role of the amino acid residue located on the fifth position before the first aspartate-rich motif of farnesyl diphosphate synthase on determination of the final product. *J Biol Chem* 271(48):30748–30754.
- Ohnuma SI, Nakazawa T, Hemmi H, Hallberg A-M, Koyama T, Ogura K, Nishino T. 1996b. Conversion from farnesyl diphosphate synthase to geranylgeranyl diphosphate synthase by random chemical mutagenesis. *J Biol Chem* 271:10087–10095.
- Ohnuma SI, Suzuki M, Nishino T. 1994. Archaeobacterial ether-linked lipid biosynthetic gene. Expression cloning, sequencing, and characterization of geranylgeranyl-diphosphate synthase. *J Biol Chem* 269:14792–14797.
- Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Sun Z, Cunningham FX Jr, Gantt E. 1998. Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. *Proc Natl Acad Sci USA* 95(19):11482–11488.
- Tan RX, Lu H, Wolfender JL, Yu TT, Zheng WF, Yang L, Gafner S, Hostetmann K. 1999. Mono- and sesquiterpenes and antifungal constituents from *Artemisia* species. *Planta Med* 65(1):64–67.
- Wang C-W, Oh M-K, Liao JC. 1999. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol Bioeng* 62:235–241.
- Wang KC, Ohnuma S. 2000. Isoprenyl diphosphate synthases. *Biochim Biophys Acta* 1529(1–3):33–48.