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Authors
Mendez-Perez, D
Alonso-Gutierrez, J
Hu, Q
et al.

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Production of jet fuel precursor monoterpenoids from engineered *Escherichia coli* †

Daniel Mendez-Perez1,2, Jorge Alonso-Gutierrez1,2, Qijun Hu1,2, Margaux Molinas1,2, Edward E. K. Baidoo1,2, George Wang1,2, Leanne Jade G. Chan1,2, Paul D. Adams1,3, Christopher J. Petzold1,2, Jay D. Keasling1,2,4,5,6, Taek Soon Lee1,2,*

1 Joint BioEnergy Institute (JBEI), 5885 Hollis Street, Emeryville, CA 94608, USA
2 Biological Systems & Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
3 Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
4 The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Horsholm, Denmark
5 Department of Chemical & Biomolecular Engineering, University of California, Berkeley, Berkeley, CA 94720, USA
6 Department of Bioengineering, University of California, Berkeley, Berkeley, CA 94720, USA

*Corresponding author: Dr. Taek Soon Lee, Joint BioEnergy Institute, 5885 Hollis St. 4th floor, Emeryville, CA 94608, USA; Phone: +1-510-495-2470, Fax: +1-510-495-2629, E-mail: tslee@lbl.gov

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Abstract

Monoterpenes (C\textsubscript{10} isoprenoids) are the main components of essential oils and are possible precursors for many commodity chemicals and high energy density fuels. Monoterpenes are synthesized from geranyl diphosphate (GPP), which is also the precursor for the biosynthesis of farnesyl diphosphate (FPP). FPP biosynthesis diverts the carbon flux from monoterpane production to C\textsubscript{15} products and quinone biosynthesis. In this study, we tested a chromosomal mutation of \textit{E. coli}’s native FPP synthase (IspA) to improve GPP availability for the production of monoterpenes using a heterologous mevalonate pathway. Monoterpene production at high levels required not only optimization of GPP production but also a basal level of FPP to maintain growth. The optimized strains produced two jet fuel precursor monoterpenoids 1,8-cineole and linalool at the titer of 653 mg/L and 505 mg/L, respectively, in batch cultures with 1\% glucose. The engineered strains developed in this work provide useful resources for the production of high-value monoterpenes. This article is protected by copyright. All rights reserved
Introduction

Isoprenoids are one of the largest and most diverse groups of products synthesized in nature. Monoterpene, a class of isoprenoids typically synthesized in the glandular structures of plants, are the main components of essential oils and are used in a wide variety of industrial applications including pharmaceuticals, insecticides, polymers and fragrances (Belgacem and Gandini, 2011; Edris, 2007; Koroch et al., 2007). However, the low freezing point and high energy density (due to methyl branching and cyclic structures, respectively) of monoterpenes also make them good candidates for gasoline, diesel and, in particular, jet fuel replacements (Bergman and Siewers, 2016; Gupta and Phulara, 2015). Isoprenoids are synthesized from two universal building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are condensed into geranyl diphosphate (GPP), farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP) to produce monoterpenes (C\textsubscript{10}), sesquiterpenes (C\textsubscript{15}), and diterpenes (C\textsubscript{20}), respectively. IPP and DMAPP can be synthesized from two distinct isoprenoid pathways: the mevalonate-dependent pathway (MVA pathway) and the mevalonate-independent or 1-deoxy-D-xylulose-5-phosphate pathway (DXP pathway). These pathways have been explored in two model organisms, 	extit{Saccharomyces cerevisiae} and 	extit{Escherichia coli}, for the production of various isoprenoids with value as pharmaceuticals and/or biofuels, including amorpha-4,11-diene (Martin et al., 2003; Ro et al., 2006), bisabolene (Peralta-Yahya et al., 2011), and farnesene (Wang et al., 2011).

Production of monoterpenes in 	extit{E. coli} has shown only limited success mainly due to the low intracellular levels of their precursor GPP via native DXP pathway, but in the past few years a heterologous mevalonate pathway has been engineered and optimized for the production of various monoterpenes such as limonene (Alonso-Gutierrez et al., 2013; Alonso-Gutierrez et al., 2015), sabinene (Zhang et al., 2014), pinene (Sarria et al., 2014) and geraniol (Zhou et al., 2014). In these works, the engineering efforts for monoterpane production in 	extit{E. coli} focused on balancing the mevalonate pathway to prevent accumulation of toxic intermediates and maximize GPP availability for
cyclization by simply coexpressing GPP synthases and monoterpane synthases. It is interesting, however, that there has been no work reported on preventing the consumption of GPP by *E. coli*’s native metabolism, which can limit the availability of GPP and eventually the production of monoterpenes. In *E. coli*, GPP is used for the biosynthesis of FPP, which is the precursor for the biosynthesis of undecaprenyl phosphate (C$_{55}$-P), a carrier lipid essential for the biosynthesis of peptidoglycan and other cell-wall polysaccharide components (Bouhss et al., 2008) (See Figure 1a). Therefore, preventing consumption of GPP by this competing pathway (i.e. FPP formation) could be beneficial for the production of monoterpenes.

In this study, we engineered a heterologous mevalonate pathway in *E. coli* for the production of monoterpenes by optimizing GPP levels and minimizing the formation of FPP. With this purpose, we introduced a mutation in *E. coli*’s genomic copy of the FPP synthase (IspA) and investigated changes in monoterpane titers as well as its effect on GPP and FPP levels. As monoterpane targets we chose 1,8-cineole, limonene, pinene, and linalool, which are potential precursors for high energy density molecules similar to conventional petroleum-derived jet fuels (Bergman and Siewers, 2016). From metabolomics analysis, we found that both GPP and FPP levels are important for monoterpane production and through a series of engineering efforts we achieved production of 653 mg/L for 1,8-cineole and 505 mg/L for linalool.

**Materials and Methods**

**Plasmids and strain construction**

*E. coli* DH10B was used as the host for cloning, and *E. coli* DH1 was used as the host for monoterpane production. Plasmids and strains used in this study are described in Table 1. For construction of plasmid JBEI-15079, used for the construction of strain DH1* (JBEI-15051), the plasmid pTrc99A was digested with EcoRI and HindIII and ligated with the 3’-end of a PCR-amplified *E. coli* ispA gene containing the mutation S81F (TTT instead of TCA), and the kanamycin cassette flanked by FRT sites (amplified from plasmid pKD4). The plasmid was linearized and integration was...
performed by λ-red recombination using plasmid pKD46 (Datsenko and Wanner, 2000). The Km′ resistance marker, located between two FRT sites and downstream of ispA, was removed by transforming cells with plasmid pCP20 and growing at 43°C (to induce FLP recombinase expression and select for loss of pCP20). The amino acid sequences of the cineole and linalool synthases were back translated, codon optimized and synthesized by IDT Inc. (Coralville, IA); the terpene synthase and GPPS were then cloned using the Gibson assembly method (Gibson et al., 2009) into the plasmid JBEI-3122 to create the 1P (one plasmid) system (resulting in plasmids JBEI-15048, JBEI-15049, JBEI-15064, JBEI-15063) or into the pTrc99a plasmid (resulting in plasmids JBEI-15066, JBEI-15065, JBEI-15062). In order to test for monoterpene production using the 2P (two plasmid) systems, the pTrc99A-GPPS-TS plasmids were co-transformed with plasmid JBEI-3122 or JBEI-3085 (for pinene production). The ispA gene and ispA* gene (S81F mutant) were amplified from DH1 or DH1* cells and cloned downstream of the terpene synthase to create plasmids JBEI-15060 and JBEI-15241.

**Monoterpene production and GC-MS analysis**

Starter cultures were grown overnight in LB at 37°C in rotary shakers at 200 rpm (Kühner, Basel, Switzerland). Production cultures were inoculated at an optical density (OD$_{600\text{nm}}$) of 0.1 and grown at 30°C EZ-Rich defined medium (Teknova, Hollister, CA) supplemented with 1% glucose and appropriate antibiotics (30 μg/mL for chloramphenicol and 100 μg/mL for ampicillin). The cultures were induced at an optical density measured at a wavelength of 600 nm (OD$_{600\text{nm}}$) 0.8 with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Samples were taken at 24 hr and 48 hr for monoterpenes production, OD$_{600\text{nm}}$ measurements and metabolomics analysis while protein measurements were performed at 24 hr during late exponential phase. OD$_{600\text{nm}}$ measurements were performed on a UV–vis spectrophotometer (Beckman, Fullerton, CA) operating at 600 nm. For production assays, a dodecane layer (10% by volume) was added to the culture upon induction to trap the monoterpenes (Anthony et al., 2009). Samples were removed from the dodecane layer (100 μL) and centrifuged at room temperature, and 10 μL of the organic phase was added to 990 μL of ethyl acetate (Sigma-Aldrich).
1 μL of the sample was injected into an Agilent 6890 series gas chromatograph (GC) equipped with an Agilent 5973 mass selective (MS) detector and an Agilent J&W DB-5ms capillary column (30 m×250 μm×0.25 μm thickness, Agilent). The GC oven temperature program was as follows: 80°C for 1 min, a ramp of 20°C/min to 120°C, a ramp of 50°C/min to 250°C. The MS was operated in selected ion monitoring (SIM) mode scanning for the molecular ions at 93, 121 and 136 m/z. Commercial monoterpenes (1,8-cineole, linalool, limonene and β-pinene (Sigma-Aldrich)) were used as standards to generate standard curves for obtaining production titers after normalizing integrated areas to an internal standard (β-pinene was used as internal standard for the quantification of 1,8-cineole, linalool and limonene; limonene was used as internal standard for the quantification of β-pinene). β-pinene was used as a pinene standard to quantify both α-pinene and β-pinene and the titers presented in this work are the sum of the titers for both isomers.

**Proteomics and metabolomics**

For proteomics analysis 1.5 mL of culture were collected at 24 hr and centrifuged at 8,000×g (at 4°C). Supernatant was decanted and the pellets were stored at -80°C. Samples were prepared as described previously (Batth et al., 2012; Redding-Johanson et al., 2011). Peptide samples were analyzed on an Agilent 6460 Triple Quadrupole mass spectrometer coupled to an Agilent 1290 liquid chromatography system (Agilent Technologies, Santa Clara, CA) operating in normal flow mode at 0.40 mL/min. The peptides (20 μg) were separated on an Ascentis Express Peptide C18 column (2.7 μm particle size, 160 Å pore size, 5 cm length x 2.1 mm i.d.) coupled to a guard column (2.7 μm particle size, 160 Å pore size, 5 mm x 2.1 mm i.d.) operating at 60°C. A 13-minute method with the following gradient was utilized: 95% Buffer A (0.1% formic acid), 5% Buffer B (98% acetonitrile, 0.1% formic acid) was held for 0.20 minutes. Buffer B was increased to 35% over 5.70 minutes, followed by an increase to 90% B in 0.30 minutes, where it was held for 2 minutes. Buffer B was then decreased to 5% in 0.50 minutes, where it was held for 4.5 minutes to re-equilibrate the column. The peptides were ionized by an Agilent Jet Stream ESI source operating in positive-ion mode with the following
source parameters: Gas Temperature = 250ºC, Gas Flow = 13 L/min, Nebulizer Pressure = 35 psi, Sheath Gas Temperature = 250ºC, Sheath Gas Flow = 11 L/min, Nozzle Voltage = 0 V, Chamber Voltage = 3500 V. The data were acquired using Agilent MassHunter, version B.06.00 and analyzed using Skyline version 3.5.

For metabolite analysis, 3 ml of culture were collected at 24 or 48 hr and centrifuged at 4ºC, quenched with 250 mL of methanol, vortexed and stored at -20ºC. For sample preparation, 250 µL of water were added to the samples and the supernatant was collected after 5 min centrifugation at 4ºC. The suspension was filtered through a Millipore™ Amicon Ultra 3 kDa cut-off filter at 14000xg for 60 min at 4ºC. 500 µL of water were added and samples were frozen with liquid nitrogen and lyophilized overnight. Samples were reconstituted in 90 µL acetonitrile-water (60:40) prior to analysis. Intracellular concentrations of GPP and FPP were measured by liquid chromatography mass spectrometry and converted into estimated intracellular concentrations as described elsewhere (George et al., 2014; Weaver et al., 2014).

Results and discussion

Pathway construction and initial production of 1,8-cineole

To build a monoterpene production platform, we chose 1,8-cineole as our initial target monoterpene. 1,8-cineole (also known as eucalyptol) is an oxygenated monoterpene synthesized by Eucalyptus globulus and other plants from the Eucalyptus genus as a protective agent to repel insects and to prevent germination of competing plants (Franks et al., 2012; Klocke et al., 1987; Wang et al., 2009). 1,8-cineole is used in non-prescription pharmaceuticals (e.g. cough suppressant, mouthwash), fragrances and flavoring agents (Lahlou et al., 2002) and can be used as additive to prevent separation in ethanol-gasoline fuel blends (Barton and Tjandra, 1989). Also, it has been shown that 1,8-cineole can be used for the synthesis of p-cymene (Leita et al., 2010; Leita et al., 2011), an important
component of the biojet fuel blend AMJ-700 (Harvey, 2016; Ryder, 2009); p-cymene and other monoterpenoid-derived molecules allow for precise control of fuel properties for aviation applications.

Monoterpene production in *E. coli* was achieved by heterologously expressing a nine-enzyme mevalonate pathway organized in three operons expressed in a single plasmid (See 1P system in Figure 1b); this arrangement has been shown to result in high production of limonene (Alonso-Gutierrez et al., 2013). The *atoB* (acetyl-CoA acetyltransferase from *E. coli*), *HMGS* (hydroxymethylglutaryl-CoA synthase from *Staphylococcus aureus*) and *HMGR* (hydroxymethylglutaryl-CoA reductase from *Staphylococcus aureus*) genes were placed under control of a LacUV5 promoter, the *MK* (mevalonate kinase from *S. cerevisiae*), *PMK* (phosphomevalonate kinase from *S. cerevisiae*), *PMD* (phosphomevalonate decarboxylase from *S. cerevisiae*) and *idi* (isopentenyl diphosphate isomerase from *E. coli*) genes were placed under control of a trc promoter, and the *GPPS* (GPP synthase from *Abies grandis*) and the terpene synthase (TS) genes were placed under control of trc promoter. Several terpene synthases from fungal, plant and bacterial origin have been reported to produce 1,8-cineole (Demissie et al., 2012; Desautels et al., 2009; Kampranis et al., 2007; Lis-Balchin, 2002; Nakano et al., 2011; Shaw et al., 2015). In this study we tested two cineole synthases (CSs); one from the bacterium *Streptomyces clavuligerus* (CS*Str*) (Nakano et al., 2011) and the other from the fungus *Hypoxylon sp.* (CS*Hyp3*) (Shaw et al., 2015), and they both have been reported to produce 1,8-cineole as their main product. The cineole synthases were cloned into the 1P system (See Figure 1b), and the resulting plasmids (JBEI-15048 or JBEI-15049) were transformed into DH1 cells and tested for production in EZ rich defined media with 1% glucose. Induction at various optical densities (OD$_{600}$nm) (0.2, 0.8, 1.2, 2.5) and inducer concentrations (25, 100, 500 µM of IPTG) was tested; although higher titers were observed at higher IPTG concentrations, OD$_{600}$nm of induction had a more significant effect on titers (lower titers were observed at OD$_{600}$nm of 2.5 and 0.2). Induction at OD$_{600}$nm of 0.8 with 500 µM IPTG resulted in the highest titers, 228 mg/L using the bacterial cineole synthase (strain CD1P) and 200 mg/L using the fungal cineole synthase (strain C$_{Hyp}$D1P) (see Table 1 for detailed description of strain
names). Although some of the experiments presented in the next sections were carried out using both cineole synthases, only the results using the bacterial cineole synthase are shown since higher titers were consistently observed when this terpene synthase was used (see supplementary Table S1 for results using CS_{Hyp3}).

Principal component analysis of proteomics (PCAP) has been used to show that production of limonene in *E. coli* at high levels requires higher limonene synthase expression levels with respect to the rest of the pathway proteins, particularly with respect to the top portion of the pathway (Alonso-Gutierrez et al., 2015). In order to test if the same approach could be used for 1,8-cineole production, a second plasmid containing an additional copy of the CS (plasmid JBEI-15068) was transformed together with plasmid JBEI-15048 (resulting in strain CD1P-CS). Production titer was improved to 305 mg/L, a 33% increase with respect to strain CD1P. The three operons containing the mevalonate pathway genes can also be expressed from two plasmids as shown in Figure 1b (system 2P) where the top and bottom portions of the mevalonate pathway are in one plasmid and the GPPS and CS are in the second plasmid containing a high-copy origin. This arrangement is expected to provide high expression levels for both GPPS and CS and to be less sensitive to small variations in the induction conditions (Alonso-Gutierrez et al., 2015). The second plasmid was constructed and tested for production as described before. As can be seen in Figure 2a, this strain (CD2P) produced 550 mg/L of 1,8-cineole at 48 hr, a significant increase from previous strains.

*Engineering of ispA to reduce conversion of GPP to FPP*

Monoterpene production in *E. coli* has mainly focused on increasing the availability of GPP, the substrate for the monoterpene synthases, by balancing the bottom and top portions of the mevalonate pathway in different plasmid arrangements leading to significant increases in production (Alonso-Gutierrez et al., 2013; Liu et al., 2016; Zhang et al., 2014). To our knowledge, however, no engineering efforts have been applied to prevent the consumption of GPP by *E. coli*’s native metabolism. Prenyl diphosphate synthases catalyze the sequential condensation reactions of IPP with different lengths of
allylic diphosphates to produce linear isoprenoid derivatives including GPP, FPP, GGPP, etc. In *E. coli*, *ispA* encodes for a farnesyl diphosphate synthase that can utilize IPP and either DMAPP or GPP as substrates, generating GPP or FPP, respectively. Since IspA has higher affinity towards GPP over DMAPP to generate FPP (Fujisaki et al., 1990), we hypothesized that elimination of this competing pathway (FPP biosynthesis) may be beneficial for monoterpene production. However, *ispA* is an essential gene in *E. coli*, and it is not possible to completely eliminate the biosynthesis of FPP. Therefore, we investigated a different strategy. It has been shown that the substrate specificity of IspA can be altered by introducing a mutation in Ser81 to Phe (S81F), resulting in an enzyme that synthesizes GPP as the main product while FPP synthase activity is significantly reduced (Ohnuma et al., 1996; Reiling et al., 2004). We hypothesized that we could increase the GPP pool for monoterpene biosynthesis while maintaining a basal level of FPP for growth by introducing this mutation into *E. coli*’s native *ispA* in the genome (resulting in the mutant *ispA*). The mutation was introduced in *E. coli* DH1’s genome using λ-red recombination (resulting in strain DH1*). Metabolomics analysis showed that GPP levels in the DH1* strain with the mevalonate pathway expressed from a single plasmid (strain CD*1P) were higher than those in strain CD1P as expected from our strain design. GPP levels of the mutant strain were more than double at 24 hr and about 40% higher at 48 hr compared to the strain with wild type *ispA* (see Figure 2c). It is interesting to note that strain CD1P showed significant accumulation of FPP both at 24 hr (46 mM) and 48 hr (58 mM), while no FPP was observed in strain CD*1P. From targeted proteomic analysis, no significant difference in the proteomic profile of all the MVA pathway proteins was observed between the CD1P and CD*1P strains (see supplementary Figure S1). These results confirm that the mutation of the genomic *ispA* to *ispA* indeed improves the availability of GPP and decreases the flux towards FPP biosynthesis. As can be seen in Figure 2a, production of 1,8-cineole reached 357 mg/L from the strain CD*1P (see Figure 2a). This represents a titer increase of more than 50% with respect to the titer of strain CD1P. The final OD$_{600nm}$’s were compared for these strains and no significant difference was observed (Figure 2b).
Interestingly, when the mevalonate pathway was expressed from two plasmids in DH1* cells (strain CD*2P), very few colonies were observed on the plates and none of these colonies produced 1,8-cineole when tested for production (more than 10 colonies were tested from multiple transformations). Also, the final OD$_{600\text{nm}}$ for this strain was lower than those of any other strains both at 24 and 48 hr (see Figure 2b). GPP and FPP were not detected when cultures from the strain CD*2P were analyzed at 24 and 48 hrs; in fact, none of the intermediates in the mevalonate pathway were detected for this strain. Proteomic analysis showed that the levels of most MVA pathway proteins were very low in the CD*2P strain compared to all the other strains, and especially the first three proteins of the pathway (AtoB, HMGS and HMGR) were expressed in extremely low levels (see Figure 3). In addition, restriction digest analysis of the plasmids isolated from strain CD*2P showed that modifications in the plasmid containing the mevalonate pathway genes (JBEI-3122) might be the cause for the lack of 1,8-cineole production in this strain (see supplementary Figure S2). These results explain why no 1,8-cineole was detected in this strain. All the MVA pathway proteins were detected when the same plasmids (JBEI-3122 and JBEI-15065) were used in DH1 cells (see strain CD2P in Figure 3).

**Optimization of 1,8-cineole production**

As previously reported, IspA(S81F) is an enzyme that preferentially synthesizes GPP instead of FPP (Reiling et al., 2004), and we expected that the strain with this mutation (DH1*) would be more efficient for monoterpenes production than the wild type strain due to the increased availability of GPP. However, this was proven true only in the case when the mevalonate pathway was expressed from a single plasmid (strain CD*1P). When the mevalonate pathway was expressed from two plasmids (strain CD*2P), 1,8-cineole production was not detected. It is important to note that FPP is essential for the biosynthesis of undecaprenyl phosphate (C$_{55}$-P), which is a carrier lipid required for the biosynthesis of peptidoglycan and other cell-wall polysaccharide components (Bouhss et al., 2008) (See Figure 1a). Therefore, it is possible that high level expression of the monoterpenic synthase in the strain DH1*, a
strain that already shows limited FPP biosynthesis, may result in selective pressure against monoterpenoid production due to low FPP levels when the 2P system is used. This explains the lower OD₆₀₀nm observed for this strain too (see Figure 2b). In order to test if increasing FPP levels would alleviate selective pressure against production and improve growth, an extra copy of the mutant ispA was introduced in the plasmid with the GPPS and CS (see 2P* in Figure 1b) and the resulting plasmid was transformed into DH1* (resulting in strain CD*2P*). As can be seen in Figure 2d, we were able to detect FPP in strain CD*2P*; higher OD₆₀₀nm was observed in this strain compared to strain CD*2P (see Figure 2b), and 1,8-cineole production was also detected. Although the overexpression of the mutant ispA from the plasmid seems to provide the FPP levels required for preventing selective pressure against production, the 1,8-cineole titer was still lower than that of strain CD2P (317 mg/L for strain CD*2P* vs 550 mg/L for strain CD2P). Metabolomics analysis showed that overexpression of the mutant ispA from the plasmid also contributes significantly to the biosynthesis of GPP; as can be seen in Figure 2c, high GPP levels were observed for strain CD*2P* even after 48 hrs.

Accumulation of GPP in strain CD*2P* might indicated suboptimal pathway performance, therefore we hypothesized that production of 1,8-cineole could be improved by reducing the accumulation of this metabolite. To reduce the accumulation of GPP, two strategies were explored. The first strategy was to increase the consumption of GPP by expressing an additional copy of the cineole synthase (see 2Pc* in Figure 1b), which resulted in strain CD*2Pc*. The second strategy was to reduce the biosynthesis of GPP by using DH1 cells instead of DH1*, resulting in strain CD2P*. Both strategies resulted in 1,8-cineole production improvement over the production from strain CD2P after 48 hr (see Figure 2a and Table S1). The 1,8-cineole titers improved to 629 mg/L for the strain CD*2Pc* and 653 mg/L for strain CD2P*, which are a 14% and 19% improvement over the strain CD2P, respectively.

It is worth noting that the only difference between the strain CD2P and the strain CD2P*, which showed the highest titer for 1,8-cineole, is the extra copy of the mutant ispA gene added in the plasmid
containing the GPPS and CS. From targeted proteomics analysis, we confirmed that the pathway protein profiles of these two strains are very similar except for the level of IspA (see Figure 3), which was higher in the strain CD2P* (the strain with the extra copy of the ispA mutant). Metabolomics analysis showed that FPP levels are slightly lower in strain CD2P* at 48 hrs compared to that of CD2P. On the other hand, GPP levels are higher in the CD2P* strain compared to the CD2P strain (particularly at 48 hr where there is a 2-fold increase) but GPP does not accumulate to high levels as in the case of the strain CD*2P* (see Figure 2c). Higher 1,8-cineole production in the CD2P* strain can therefore be explained by higher GPP levels when an additional copy of the mutant ispA is expressed from the plasmid. However, it is important to note that when the mutant ispA in the plasmid JBEI-15060 is replaced with the wild type ispA (strain CD2P-IspA) or when an extra copy of Abies grandis GPPS, which exclusively produces GPP without producing FPP (Burke and Croteau, 2002), is expressed from a second plasmid along with 1P plasmid JBEI-15048 (strain CD1P-G), a significant 1,8-cineole titer drop was observed (See Table S1). This supports the idea that high production of 1,8-cineole requires not only higher GPP levels but also a basal level of FPP to maintain growth. Mutation of the chromosomal ispA seems to provide the strain enough FPP levels to support growth when the 1P system is used, where the CS as well as GPPS expression levels were relatively low. However, this was not the case when the mevalonate pathway is expressed from the 2P system, in which the CS and GPPS are expressed at high levels from a high copy plasmid. In this case, in order to maintain growth and prevent selection pressure against monoterpene production it was necessary to express an additional copy of the mutant ispA from a plasmid.

Production of other jet fuel precursor monoterpenes

The platforms and strategies developed in previous sections could be used for the production of other monoterpenes by expressing different monoterpene synthases. Among various monoterpenes, we were interested in pinene, limonene and linalool, which have been explored as precursors for alternative aviation fuels (Harvey et al., 2010; Meylemans et al., 2011; Tracy et al., 2009). The low
freezing point of hydrogenated limonene makes it a good candidate to enhance cold weather performance in jet fuel mixtures (Tracy et al., 2009). Hydrogenated pinene dimers have been synthesized via chemical dimerization and shown to have volumetric energy and net heat of combustion comparable to the aviation turbine fuel JP-10 (Harvey et al., 2010). Linalool has also been used as a precursor for the high-density fuel RJ-4 (a liquid rocket propellant used in missiles and a component of jet fuel) via olefin metathesis, dehydration and dimerization (Meylemans et al., 2011).

The limonene synthase from Mentha spicata (Hyatt et al., 2007), the pinene synthase from Abies grandis (Bohlmann et al., 1997) and a linalool synthase from Mentha citrate (Crowell et al., 2002) were tested for monoterpen production using the 1P and 2P systems in both DH1 and DH1* cells. When the limonene synthase was tested using the 1P system, a higher titer of limonene was observed in the strain with the mutant ispA compared to the one with the wildtype ispA (123 mg/L in strain LmD1P vs 214 mg/L in strain LmD*1P, see Table S1). Similar to 1,8-cineole production in strain CD*2P, no limonene production was observed from the strain with the chromosomal mutation on the ispA when the mevalonate pathway was expressed from two plasmids (strain LmD*2P). In the case of the pinene producing strains, there was no titer improvement in the DH1* strain with the 1P system, in fact lower titer was observed in the strain with the mutant ispA (19 mg/L for strain PD1P vs 9 mg/L for strain PD*1P, see Table S1). When the 2P system was used, however, there was a two-fold improvement in pinene production in the strain with the mutant ispA (14 mg/L for the PD2P strain vs 27 mg/L for the PD*2P strain), which is comparable to the titer reported in a previous study using the same pinene synthase (Sarria et al., 2014). It is important to note that pinene was the only monoterpen we could detect when the 2P system was used in DH1 with the ispA mutant (strain PD*2P). Since pinene titers were significantly lower than those of other monoterpens (more than one order of magnitude lower), it is possible that GPP consumption for pinene production using the 2P system may not be high enough to cause FPP depletion in DH1* cells.
The highest improvement in monoterpane production using the strain with the mutant ispA was observed for linalool (see Figure 4). Several terpene synthases from fungal, plant and bacterial origin have been reported to produce linalool (Crowell et al., 2002; Dudareva et al., 1996; Jia et al., 1999; Landmann et al., 2007; Pichersky et al., 1995) but only a few studies have shown microbial production of this molecule at low titers (Amiri et al., 2016; Thanasomboon et al., 2012). We cloned the truncated 3R-linalool synthase, in which the plastidal transit peptide was deleted, from Mentha citrate (Crowell et al., 2002) into the 1P, 2P and 2P* systems, and they were transformed and tested for production in both wild type and the ispA mutant DH1 host strains. The results for production are shown in Figure 4.

The highest titer was observed in the strain LD*1P at 505 mg/L. Similar to the results from the 1,8-cineole and limonene production strains, we observed higher production of linalool in DH1 cells with the mutant ispA (strain LD*1P) compared to the wild type DH1 host (strain LD1P) when the 1P system was used, and no production was observed when the 2P system is used in the DH1 host with the mutant ispA (strain LD*2P). Metabolomics analysis showed that no detectable level of GPP and FPP were produced from this strain (LD*2P) at 24 and 48 hr (see supplementary Figure S3). Linalool production was restored when an additional copy of the mutant ispA was included in the plasmid containing the GPPS and linalool synthase (strain LD*2P*), but no GPP accumulation was observed for this strain as it was the case when the cineole synthase was used. It is interesting to note that higher titers of linalool were observed when the linalool synthase was expressed from a medium copy plasmid (1P system) and not from a high copy plasmid (2P system) as it was the case for the 1,8-cineole producing strains.

Differences in the kinetic parameters and solubilities of the monoterpane synthases might explain the different expression levels require for monoterpane production at high titers, but more research would be needed to confirm this hypothesis.

**Conclusions**

Monoterpenes and monoterpane-derived molecules are promising alternatives to aviation fuels; their properties, such as carbon chain length, heat of combustion and freezing point, are very similar to
conventional petroleum-derived jet fuels (Bergman and Siewers, 2016). In fact, a biojet fuel (AMJ-700) developed by Amyris Inc. has already been used in a demonstration flight without requiring any changes to the aircraft (Amyris, 2012). Monoterpenes (that make up 60% of this jet fuel mixture) were essential for meeting the engine performance requirements and strict physicochemical specifications (Ryder, 2009).

In this study, we developed an E. coli platform for the production of monoterpenes using a heterologously expressed mevalonate pathway and a chromosomal mutation on E. coli’s native FPP synthase (IspA) to increase availability of GPP. The platform was successfully demonstrated for the production of various monoterpenes including 1,8-cineole and linalool, two important monoterpenes that can be used as precursors of high energy density fuels. Metabolomics analysis showed that GPP levels were higher when the IspA mutant was used, with a corresponding decrease in FPP levels. However, using a strain with limited FPP biosynthesis may resulted in selective pressure against monoterpane production when the terpene synthases are expressed from a high-copy plasmid. Therefore, production of monoterpenes at high levels also requires a basal level of FPP to maintain growth. After optimization the engineered strains produced 653 mg/L of 1,8-cineole and 505 mg/L of linalool, a 30-fold and 5-fold improvement, respectively, from previous reports.

Acknowledgments

We would like to thank Charles Denby for his valuable suggestions selecting the linalool synthase used in this study, and Noreen Wauford and Victor Tieu for suggesting some of the cineole synthases tested. This work was part of the DOE Joint BioEnergy Institute (http://www.jbei.org) supported by the US Department of Energy, Office of Science, Office of Biological and Environmental Research, through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the US Department of Energy.

Author contributions
DMP, JAG, and TSL designed the experiments. DMP, JAG, QH, MM, EEKB, GW, LJGC, PDA, CJP performed the experiments and analyzed data. DMP, JDK and TSL wrote the manuscript.

**Competing financial interests:** JDK has financial interest in Amyris, LS9, and Lygos.

**References**


Table 1. Plasmids and strains used

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a MTSA and MevT operons contain genes for the biosynthesis of mevalonate from acetyl-CoA (AtoB, HMGS, HMGR under control of a LacUV5 promoter); MBI operon contains genes for the biosynthesis of IPP and DMAPP from mevalonate (MK, PMK, PMD and Idi under control of a trc promoter); T1: double terminator followed by Ptrc; T1002: terminator. b Strain names for monoterpane production are prepared systematically. The first part of the strain name describes the product (C: cineole, P: pinene, Lm: limonene, L: linalool, C_Hyp: cineole using the fungal cineole synthase), the second part describes the host strains (D: DH1 vs D*: DH1*), the third part describes the system (1P, 2P, 2P*, or 2Pc* as shown in Figure 1b), and the last part describes any additional modification (CS: additional cineole synthase, IspA: additional IspA, G: additional GPPS). CS_St.: cineole synthase from Streptomyces clavuligerus, AgGPPS-(GSG)2-AgPS: genes encoding for A. grandis GPPS-PS protein fusion.
**Figure legends**

**Figure 1.** Heterologous mevalonate pathway used for the production of monoterpenes in *E. coli* (a) Monoterpene biosynthetic pathway. (b) Plasmids used in this study. TS: Terpene synthase, *ispA*:* gene encoding for IspA(S81F), C55-P: undecaprenyl phosphate, AtoB: acetyl-CoA acetyl transferase, HMGS: hydroxymethylglutaryl-CoA synthase, HMGR: hydroxymethylglutaryl-CoA reductase, MK: mevalonate kinase, PMK: phosphomevalonate kinase, PMD: phosphomevalonate decarboxylase, Idi: isopentenyl diphosphate isomerase, GPPS: GPP synthase, CS: cineole synthase.

**Figure 2.** Production of 1,8-cineole in the engineered strains (a) 1,8-cineole titers at 24 and 48 hours. (b) Optical density. (c) Intracellular geranyl diphosphate (GPP) measured at 24 and 48 hr. (d) Intracellular farnesyl diphosphate (FPP) measured at 24 and 48 hr. 1,8-cineole, GPP and FPP levels for strain DH1 (without plasmids) are not displayed as they were below detection level. Cultures were grown at 30°C in EZ-rich defined medium with 500 µM IPTG and 10% dodecane overlay. Error bars represent the standard deviation from 3 biological replicates.

**Figure 3.** Proteomics comparison. Areas of the proteins from the mevalonate pathway were normalized to the area of chloramphenicol acetyltransferase; IspA corresponds to the area of both wildtype IspA and the mutant IspA(S81F). Results are the average of three biological replicates, with error bars representing the standard deviation.

**Figure 4.** Production of linalool in the engineered strains. Cultures were grown at 30°C in EZ-rich defined medium with 500 µM IPTG and 10% dodecane overlay. Error bars represent the standard deviation from 3 biological replicates.
Figure 2

(a) 1,8-cineole

(b) OD<sub>600nm</sub>

(c) GPP

(d) FPP

Legend:
- Chromosomal <i>ispA</i>: wt, <i>ispA</i>*, wt, <i>ispA</i>*
- Plasmid origin for GPPS, CS genes: p15A, p15A, ColE1, ColE1, ColE1
- <i>ispA</i> copy in plasmid: -, -, +, +, +

Y-axis labels:
- 1,8-cineole production (mg/mL)
- Optical density (OD<sub>600nm</sub>)
- Intracellular GPP (mM)
- Intracellular FPP (mM)
Figure 4

Linalool production (mg/L)

Columns represent different conditions or treatments, with error bars indicating variability. The bars are labeled with abbreviations such as LD1P, LD*1P, LD2P, LD*2P, LD2P*, and LD*2P*.