

Carbon partitioning to the terpenoid biosynthetic pathway enables heterologous β -phellandrene production in *Escherichia coli* cultures

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Abstract *Escherichia coli* was used as a microbial system for the heterologous synthesis of β -phellandrene, a monoterpene of plant origin with several potential commercial applications. Expression of *Lavandula angustifolia* β -phellandrene synthase (*PHLS*), alone or in combination with *Picea abies* geranyl-diphosphate synthase in *E. coli*, resulted in no β -phellandrene accumulation, in sharp contrast to observations with *PHLS*-transformed cyanobacteria. Lack of β -phellandrene biosynthesis in *E. coli* was attributed to the limited endogenous carbon partitioning through the native 2-C-methylerythritol-4-phosphate (MEP) pathway. Heterologous co-expression of the mevalonic acid pathway, enhancing cellular carbon partitioning and flux toward the universal isoprenoid precursors, isopentenyl-diphosphate and dimethylallyl-diphosphate, was required to confer β -phellandrene production. Differences in endogenous carbon flux toward the synthesis of isoprenoids between photosynthetic (*Synechocystis*) and non-photosynthetic bacteria (*E. coli*) are discussed in terms of differences in the regulation of carbon partitioning through the MEP biosynthetic pathway in the two systems.

Keywords β -Phellandrene · Isoprenoid biosynthetic pathway · Metabolic engineering · Monoterpene biosynthesis

Abbreviations

DCW	Dry cell weight
DMAPP	Dimethylallyl-diphosphate
GPSS	Geranyl-diphosphate synthase
IPP	Isopentenyl-diphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
MEP	2-C-methyl-erythritol-4-phosphate
MVA	Mevalonic acid
OD	Optical density
β -PHL	β -Phellandrene
PHLS	β -Phellandrene synthase
TIR	Translation initiation region

Introduction

The monoterpene β -phellandrene ($C_{10}H_{16}$) is naturally synthesized from geranyl-diphosphate (GPP) by a number of plant species as a constituent of their essential oils. It has commercial value as a key ingredient in medical, cosmetic and cleaning products, and potentially as a fuel (Martin et al. 2012; Bentley et al. 2013; Zerbe and Bohlmann 2014). Microbial production of β -phellandrene through metabolic engineering can provide a sustainable alternative by which to meet increasing product demand from the commercial sector. Microbial systems are suitable for large-scale production, as they are cultivated in bioreactors that satisfy containment and product sequestration requirements.

Heterologous biosynthesis of β -phellandrene was first engineered in a photosynthetic microorganism, the cyanobacterium *Synechocystis* (Bentley et al.

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2013), taking advantage of the cell's photosynthesis-driven metabolism. A positive aspect of microbial β -phellandrene production is the spontaneous and quantitative separation of the molecule from the biomass and the liquid culture (Bentley et al. 2013; Formighieri and Melis 2014). Floating of the β -phellandrene on the surface of the liquid culture facilitates segregation and harvesting, a parameter that weighs heavily on the economics of a microbial production system. Further, β -phellandrene separation from the biomass alleviates potential inhibitory or toxic effects of the molecule on cellular metabolism (Lindberg et al. 2010; Dunlop et al. 2011). However, although *Synechocystis* is a well-studied model microorganism, over-expression of transgenic proteins, to a level that can be visually detected in Coomassie-stained SDS-PAGE of total protein extract, is rarely achieved. This is an important consideration, as low concentration of the transgenic terpene synthase is a factor limiting β -phellandrene production rate and yield (Formighieri and Melis 2014).

Different biological systems employ two independent metabolic pathways by which to generate the universal terpenoid precursors isopentenyl-diphosphate (IPP) and dimethylallyl-diphosphate (DMAPP). The 2-C-methylerythritol-4-phosphate (MEP) pathway, which is of prokaryotic origin and active in most bacteria and plant plastids, requires pyruvate and glyceraldehyde 3-phosphate as initial substrates for IPP and DMAPP biosynthesis. The mevalonic acid (MVA) pathway uses acetyl-CoA and operates in the cytosol of eukaryotes and archaea. *Escherichia coli* possesses the MEP pathway for the synthesis of primary terpenoids essential for cellular function, in particular for the prenylation of tRNAs and the synthesis of farnesyl diphosphate (FPP), which is used for quinone and cell wall biosynthesis (Bouhss et al. 2008; Dumelin et al. 2012). The limited number and amount of terpenoid products in *E. coli* suggest slow flux of endogenous carbon through the MEP pathway and low intracellular levels of C₅ isoprenoid precursors (IPP and DMAPP) (Carter et al. 2003). Engineering the endogenous MEP pathway to enhance flux is one strategy by which to increase IPP and DMAPP supply (Farmer and Liao 2001; Kim and Keasling 2001; Matthews and Wurtzel 2000). However, heterologous expression of the MVA pathway in *E. coli* was reported to be a superior strategy (Martin et al. 2003; Vadali et al. 2005; Yoon et al. 2009; Zurbriggen et al. 2012; Alonso-Gutierrez et al. 2013), avoiding native cellular regulatory mechanisms that apply breaks to the MEP pathway (Banerjee et al. 2013). With respect to monoterpene production, an additional issue is the apparent absence of a distinct GPP synthase in *E. coli*, further raising the question of the efficacy of this microbial system for the synthesis of monoterpenes (Carter et al. 2003).

Recent work on the synthesis of monoterpenes in *E. coli*, such as limonene, pinene, and sabinene, employed heterologous expression of the MVA pathway in combination with the heterologous expression of a geranyl-diphosphate synthase (GPPS), resulting in widely variable yields of the various monoterpenes (Alonso-Gutierrez et al. 2013; Sarria et al. 2014; Zhang et al. 2014).

In the present work, we investigated aspects of the synthesis of β -phellandrene in the chemoheterotrophic, facultative aerobic bacterium *E. coli*. Heterologous co-expression of *Lavandula angustifolia* β -phellandrene synthase (PHLS) along with *Picea abies* GPPS did not yield measurable amounts of β -phellandrene. The additional heterologous expression of the MVA biosynthetic pathway was required for β -phellandrene accumulation in the culture. Results are discussed in terms of the regulation of carbon flux through the bacterial MEP isoprenoid biosynthetic pathway, and also in terms of the plasmid- versus chromosomal-based expression of transgenes in production-type photosynthetic (e.g., *Synechocystis*) and chemoheterotrophic (*E. coli*) bacteria.

Materials and methods

Escherichia coli strains and plasmids

The *L. angustifolia* (lavender) codon-optimized *PHLS* gene (Bentley et al. 2013; Formighieri and Melis 2014, see also Supplemental materials) was cloned via NdeI and BglII restriction enzymes in the pJF plasmid (AmpR) under the control of the P_{trc} promoter and lac Operator.

Three different types of GPPS gene have been identified in plants, a heterodimeric and two homodimeric enzymes. *Picea abies* (Norway spruce) harbors two *GPPS* genes (*GPPS2* and *GPPS3*), each encoding a different homodimeric enzyme. In contrast to *GPPS3*, *GPPS2* was chosen in the present study as it clusters with other gymnosperm GPPS sequences, producing GPP as the sole in vitro product (Schmidt and Gershenzon 2008). The sequence encoding for *Picea abies* (Norway spruce) *GPPS2* was obtained from the NCBI GenBank database (EU432047.2) and codon optimized using the Gene Designer 2.0 software from DNA2.0, upon removal of 67 amino acids predicted by ChloroP to constitute the chloroplast transit peptide. The resulting *GPPS2* sequence (Supplemental material) was cloned via BglII restriction downstream of *PHLS* in the pJF plasmid in an operon configuration. The (N)6-AGGAGG-(N)6 nucleotide sequence, shown to afford efficient ribosome binding in the translation of recombinant proteins in *E. coli* (Zurbriggen et al. 2012), was used as *GPPS2* translation initiation region (TIR). *E. coli* Rosetta cells were first transformed with the pJF-*PHLS-GPPS2* plasmid, while a

second transformation event introduced the pET28a-MVA (KanR) plasmid. The latter drives expression of a super-operon containing the seven genes for expression of a complete MVA pathway, as described (Zurbriggen et al. 2012), under the control of the T7 promoter and lac Operator.

β -Phellandrene production assay in *E. coli*

A 400-mL bacterial culture, comprising minimal M9 media supplemented with 0.4 % glycerol or LB media supplemented with 1 % glucose, was grown in flasks by shaking at 180 rpm, at 37 °C until the culture reached an OD at 600 nm of 0.6. It was then cooled to room temperature and transferred to a 1-L glass reactor. Recombinant protein expression was induced with the addition of 1 mM IPTG, and the reactor was sealed and placed at 28 °C for 6 h, shaking at 60 rpm. During the first 6 h of incubation, the reactor 800 mL headspace provided the oxygen needed for the growth and productivity of the bacteria, while containing the β -phellandrene product. 10 mL of hexane were added as hydrophobic over-layer solvent on the surface of the culture (Bentley et al. 2013) and incubated at 60 rpm until the end of the batch experiment, for a total of 20-h incubation. In some experiments, incubation was extended to 72 h. Fitness and growth of *E. coli* were not impeded by the application of the thin hydrophobic layer of hexane.

The β -phellandrene product was assayed from the specific absorbance of the compound in the UV region of the spectrum, showing a primary peak at 232.4 nm in hexane. An extinction coefficient of $15.7 \text{ mM}^{-1} \text{ cm}^{-1}$ at 232.4 nm was applied (Formighieri and Melis 2014). β -Phellandrene was independently quantified by GC analysis of 1 μL of hexane extracts in which a known amount of limonene was added (62118 Sigma, (R)-(+)-limonene), serving as an internal standard for the GC injection and by comparing the corresponding peak areas in gas chromatography printouts (Shimadzu GC-2014 equipped with a Rtx[®]-5 column). We assumed that equal amounts of limonene and β -phellandrene, when injected in the GC, would generate equal peak heights in the GC printout. The following integration parameters for GC peak identification were employed: slope 100 $\mu\text{V}/\text{min}$, width 3 s. The resolution limit of the GC for detection and quantification of monoterpenes was about 10 ng/ μL . In this analysis, the temperature of the GC column was initially set at 40 °C for 3 min, followed by a two-step temperature increase, first to 75 °C (at a rate of 4 °C/min, then held at 75 °C for 8 min), and second to 250 °C (at a rate of 20 °C/min). The carrier gas (hydrogen) flow rate was 1.5 mL per min. The β -phellandrene product was chemically identified by mass spectrometry (Bentley et al. 2013).

Protein analysis of cleared lysate and pellet by SDS-PAGE and immunoblotting

At the end of the 20-h incubation period, an aliquot of 50-mL culture was pelleted and resuspended in buffer (50 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM CaCl_2 , 10 mM MgCl_2) plus protease inhibitors (1 mM PMSF, 2 mM aminocaproic acid, 1 mM benzamidine). The cell suspension was treated first with lysozyme (Thermo Scientific) then with DNaseI (from bovine pancreas, Sigma D4527) for 30 min each at room temperature. Cell disruption was achieved by sonication and by addition of 0.5 % Triton X-100 to the cell suspension. The cleared lysate was separated from the pellet by centrifugation at 21,000 g for 15 min. The pellet was resuspended in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.2) including 8 M urea. The protein profile of the cleared lysate and pellet samples was analyzed by SDS-PAGE (Bio-Rad, USA), and either visualized by Coomassie brilliant blue G staining or upon transfer to nitrocellulose membrane for reaction with specific polyclonal antibodies raised against the transgenic proteins. Specific polyclonal antibodies against the PHLS and individual enzymes of the MVA pathway were raised in rabbit, as previously described (Zurbriggen et al. 2012; Bentley et al. 2013). The synthetic peptide DDLPYIDNDDLRRG-KPTNHK was used to raise specific polyclonal antibodies in rabbit against the *Picea abies* GPPS protein (ProSci, Inc., Poway, CA 92064).

Results

Transgenes for the β -phellandrene synthase (*PHLS*), geranyl-diphosphate synthase (*GPPS*), and the entire MVA pathway were cloned in separate plasmids (Fig. 1) followed by transformation of *E. coli* Rosetta cells. Different construct combinations were applied to determine individual gene contributions to β -phellandrene biosynthesis: (i) the *PHLS* gene alone; (ii) a combination of *PHLS* and *GPPS*; (iii) the *PHLS* in combination with the MVA pathway operon; and (iv) the *PHLS* with both the *GPPS* and the MVA pathway operon. Cell biomass and β -phellandrene accumulation were pursued in batch cultures at 28 °C, as temperatures lower than 37 °C were reported to favor terpene biosynthesis in *E. coli* (Alonso-Gutierrez et al. 2013). In all cases, β -phellandrene efficiently diffused through the plasma membrane and cell wall and, because of its hydrophobicity, accumulated as a floater molecule at the surface of the culture. It was extracted upon dilution with a known amount of hexane, the latter serving as a product carrier molecule.

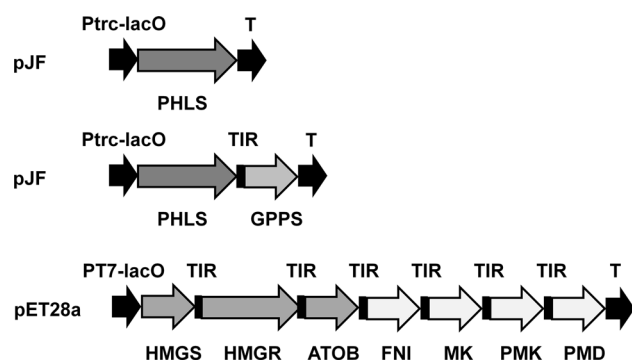


Fig. 1 Schematic of recombinant DNA constructs expressed in *E. coli* for β -phellandrene biosynthesis. PHLS from *L. angustifolia* and GPPS2 from *Picea abies* were cloned in pJF plasmid under the control of the IPTG inducible Ptrlac promoter and lac Operator. The seven genes encoding the entire MVA pathway were cloned as a superoperon in plasmid pET28a under the control of the IPTG inducible T7 promoter and lac Operator, as recently described (Zurbriggen et al. 2012). TIR1 (Zurbriggen et al. 2012) was used as translation initiation region for each gene. HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase from *E. faecalis*. HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase from *E. faecalis*. ATOB, acetyl-CoA acetyl transferase from *E. coli*. FNI, isopentenyl-pyrophosphate isomerase from *S. pneumoniae* R6. MK, mevalonic acid kinase from *S. pneumoniae* R6. PMK, phospho-mevalonic acid kinase from *S. pneumoniae* R6. PMD, di-phospho-mevalonic acid decarboxylase from *S. pneumoniae* R6

Growth of *E. coli*, PHLS protein expression, and β -phellandrene production was first assessed in minimal media (M9) supplemented with 0.4 % glycerol. The hexane over-layer was collected 20 h after induction of the transgenic protein expression, for quantification of the β -phellandrene product. Aliquots of the *E. coli* culture were also collected for biomass and protein analysis. Total cell proteins were extracted from the biomass, separated by centrifugation in cleared lysate (supernatant) and pellet and analyzed by SDS-PAGE. Presence of the 64 kD PHLS protein was visible in the Coomassie-stained gel (Fig. 2, PHLS) under the various transformant configurations employed. Transgenic PHLS accumulated mostly in the supernatant with lower levels of the PHLS also appear in the pellet (Fig. 2, PHLS). These results showed the ability of *E. coli* to over-express PHLS as a soluble enzyme.

Hexane extracts from the surface of untransformed control and different transformant cultures were obtained for β -phellandrene quantification (Fig. 3) from the specific absorbance of the solution in the UV region of the spectrum (Formighieri and Melis 2014). Figure 3 shows such absorbance spectra of hexane extracts from different transformants and untransformed controls, normalized on per g of *E. coli* dry cell weight (dcw). In this case, control cultures (Fig. 3, C) and transformants with the PHLS gene (Fig. 3, P) showed no spectrophotometric or GC evidence for the presence of β -phellandrene. We tested the hypothesis that

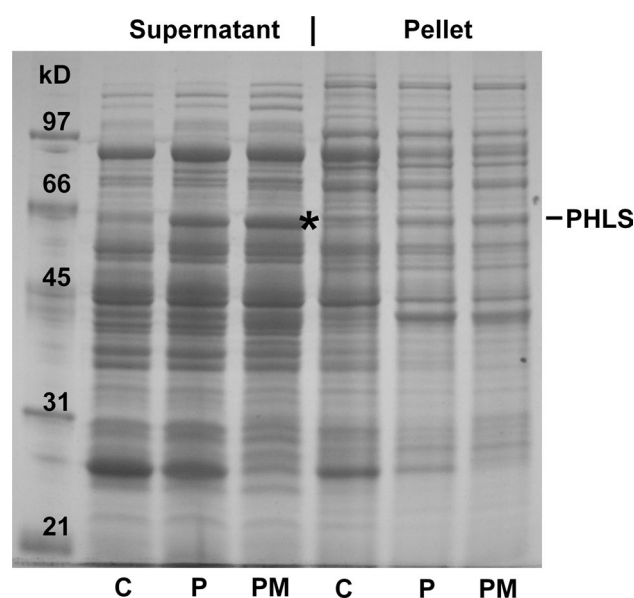


Fig. 2 Coomassie-stained SDS-PAGE of proteins from cleared lysates (supernatant) and pellet fractions of different *E. coli* transformants grown on minimal medium (M9) supplemented with 0.4 % glycerol. C, Rosetta cells serving as control. P, PHLS. PM, PHLS plus MVA pathway. Molecular weight markers were loaded on the left-most lane. The asterisk marks the electrophoretic mobility of the PHLS (64 kD) protein, which is enriched in the supernatant fraction

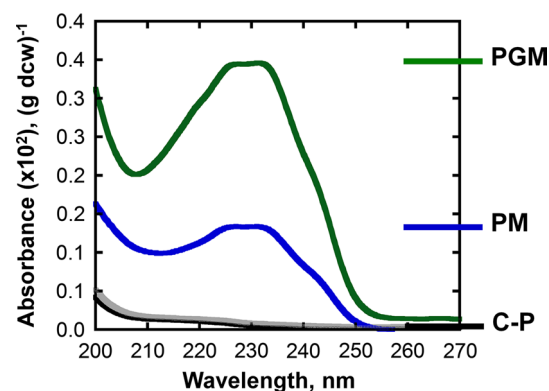


Fig. 3 Absorbance spectra of hexane extracts from *E. coli* transformants normalized on a per g dcw, following a 20 h batch incubation of the cells. *E. coli* cultures were grown in minimal media (M9) supplemented with 0.4 % glycerol. C, Rosetta cells serving as control. P, PHLS. PM, PHLS plus MVA pathway. PGM, PHLS-GPPS plus MVA pathway. The absorbance spectra are the average of at least three independent biological replicates. Variance of the spectral peak height was within ± 30 % of the reported value

slow intracellular flux to IPP and DMAPP isoprenoid precursors may impede β -phellandrene biosynthesis by heterologously expressing the MVA pathway in the cells. Co-expression of the MVA pathway with the PHLS alone, or in combination with GPPS, resulted in specific absorbance of the solution in the UV region of the spectrum, with

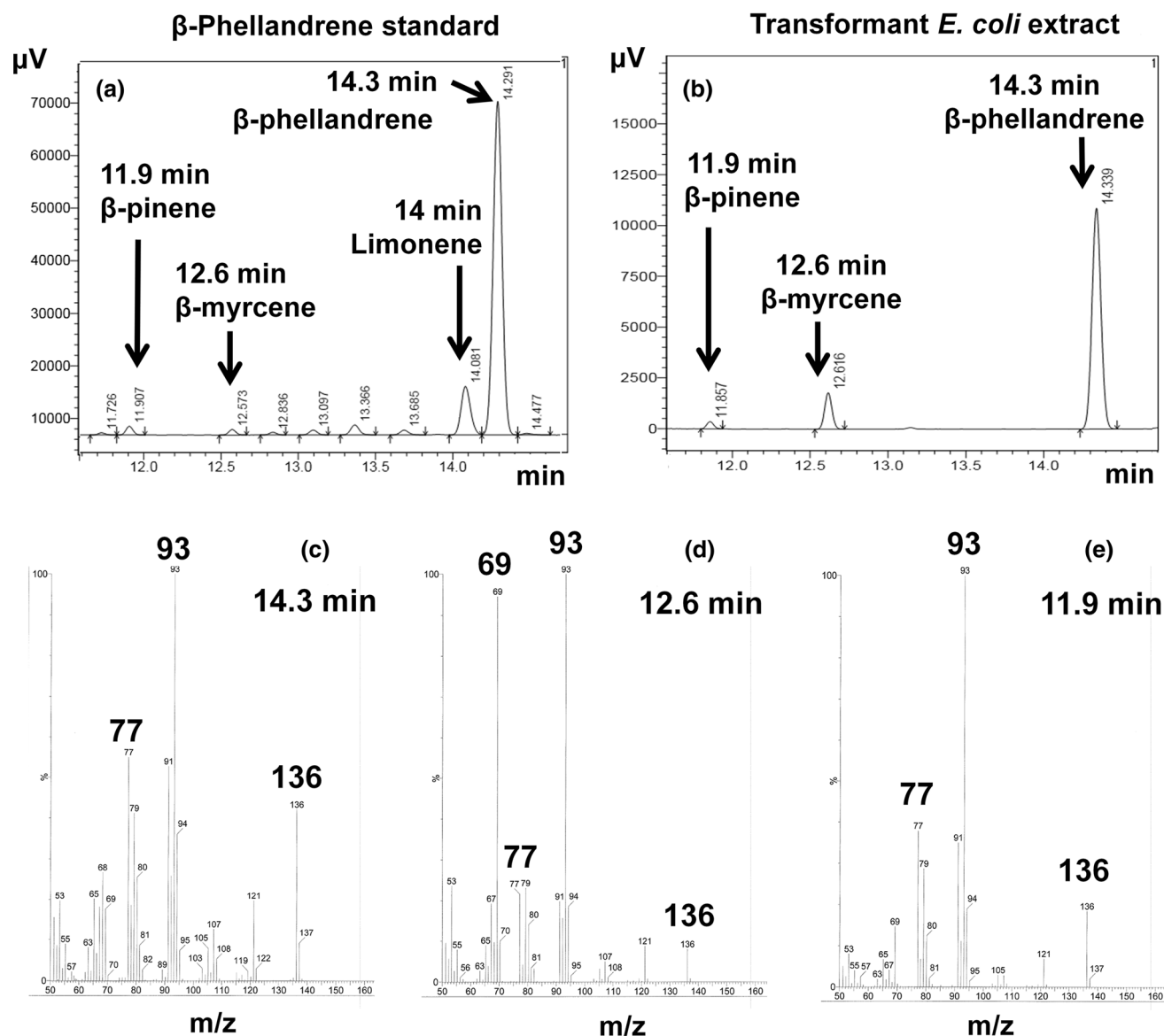


Fig. 4 GC analysis of hexane extracts from transformant *E. coli* cultures and product identification by MS. **a** GC analysis of a β -phellandrene standard (Chemos GmbH) showing a retention time of 14.3 min. The β -phellandrene standard contained other monoterpenes as impurities. Limonene (retention time of 14 min), β -myrcene (retention time of 12.6 min) and β -pinene (retention time of 11.9 min) are highlighted. **b** GC analysis of the hexane extract from PHLS+GPPS+MVA transformant *E. coli* cultures. Equivalent results were obtained with PHLS+MVA cultures. This analysis showed the presence of β -phellandrene (retention time of 14.3 min) as the major

product, along with traces of β -myrcene and β -pinene. GC analysis of hexane extracts from untransformed Rosetta (control) cells and from the PHLS transformant displayed a flat profile, showing no discernible peaks in the 5–20 min retention time region (not shown). **c** The 77, 93, 136 MS signature lines of the compound with retention time of 14.3 min, identifying the molecule as β -phellandrene. **d** MS signature lines of the compound with retention time of 12.6 min, identifying the molecule as β -myrcene. **e** MS signature lines of the compound with retention time of 11.9 min, corresponding to β -pinene

a primary peak at 232.4 nm (Fig. 3, PM and PGM), that matched the absorbance profile of a β -phellandrene standard (Formighieri and Melis 2014). Yields were estimated to be 1.1 mg β -phellandrene per g dcw (Fig. 3, PM) and 3 mg β -phellandrene per g dcw (Fig. 3, PGM).

The above spectrophotometric results were corroborated by GC–MS analysis (Fig. 4). GC analysis of the

β -phellandrene standard (Chemos GmbH) showed a primary peak with a retention time of 14.3 min (Fig. 4a), followed by minor peaks attributed to the presence of limonene (retention time of 14 min) and other impurities. GC analysis of hexane extract from *E. coli* transformants expressing the PHLS and the MVA pathway, and transformants expressing the PHLS, along with the GPPS and the MVA pathway,

yielded qualitatively similar results, showing three peaks with retention times of 11.9, 12.6, and 14.3 min (Fig. 4b). None of these peaks were detected in the untransformed (control) *E. coli*, or *E. coli* transformed with *PHLS* gene alone (not shown). MS analysis of the compound with retention time of 14.3 min showed pronounced 77, 93, 136 signature MS lines in both the β -phellandrene standard and the hexane extracts from transformant *E. coli* cultures (Fig. 4c). This MS line signature corresponds to β -phellandrene (Formighieri and Melis 2014), and clearly shows the presence of β -phellandrene as a floater molecule in some of the transformant *E. coli* cultures. MS analyses of the compound with retention time of 12.6 and 11.9 min are shown in Fig. 4d, e, respectively. Comparison of these MS lines with those available in the literature (webbook.nist.gov) identified the corresponding molecules as β -myrcene (Fig. 4d) and β -pinene (Fig. 4e). These monoterpenes are also present as impurities in the β -phellandrene standard (Fig. 4a). It is common for terpene synthases to naturally convert the substrate into multiple related products. In this respect, PHLS from *L. angustifolia* was reported to be more specific for β -phellandrene than PHLS enzymes from other plants (Demissie et al. 2011). These results support the notion that heterologously expressed PHLS from *L. angustifolia* synthesizes β -phellandrene as its major product (87 %), with a secondary synthesis of β -myrcene (11 %) and β -pinene (2 %). Lesser amounts of monoterpenes isomeric to β -phellandrene emanate from the promiscuous catalytic activity of the β -phellandrene synthase (Bohlmann et al. 1999; Schillmiller et al. 2009) or from unspecific β -phellandrene isomerization to other monoterpenes. The results further suggest that endogenous carbon partitioning to the isoprenoid biosynthetic pathway in *E. coli* is not sufficient to support the generation of β -phellandrene, regardless of the level of PHLS enzyme that may accumulate in the cell.

To ensure the validity of the above contentions, and to eliminate the source of carbon as a variable affecting the results, we repeated the above analysis upon transformation and growth of *E. coli* in LB media supplemented with 1 % glucose. Similar to the previous experiment, the hexane over-layer was collected 20 h after induction of transgenic protein expression, for quantification of the β -phellandrene product. Aliquots of the *E. coli* culture were also collected for biomass and protein analysis. Total cell proteins were extracted from the biomass, separated in cleared lysate (supernatant) and pellet, and analyzed by SDS-PAGE (Fig. 5). Presence of the 64 kD PHLS protein was visible in the Coomassie-stained gel, both in the supernatant and in the pellet (Fig. 5, PHLS), although in this case most of the PHLS pelleted upon centrifugation at 21,000 g for 15 min. Extra protein bands were detected in the supernatant of the MVA transformants (Fig. 5 PM and PGM), appearing as ~80, 40 and 35 kD proteins in Fig. 5.

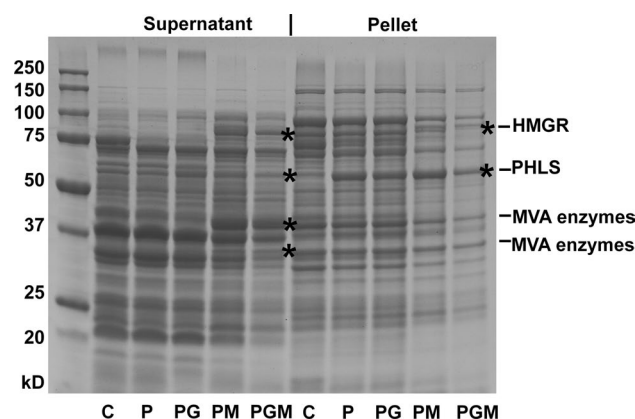


Fig. 5 Coomassie-stained SDS-PAGE of cleared lysates (supernatant) and pellet fractions of *E. coli* control and different transformants. Cultures were incubated in LB media supplemented with 1 % glucose. C, Rosetta cells serving as control. P, PHLS. PG, PHLS-GPPS. PM, PHLS plus MVA pathway. PGM, PHLS-GPPS plus MVA pathway. Molecular weights: PHLS, 64 kD; GPPS, 35 kD; HMGS, 42 kD; HMGR, 86 kD; ATOB, 40 kD; FNI, 38 kD; MK, 31 kD; PMK, 37 kD; PMD, 39 kD. Molecular weight markers were loaded on the left-most lane. Asterisks denote protein bands not present in the control, reflecting expression of transgenic proteins

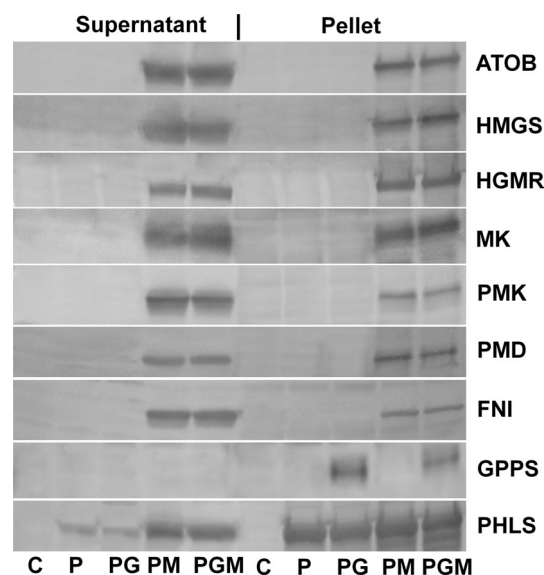


Fig. 6 Western blot analysis of supernatant and pellet fractions as shown in Fig. 5, probed with specific polyclonal antibodies directed against the corresponding transgenic proteins

Expression of the transgenic proteins was further assayed in this case by Western blot analysis with specific polyclonal antibodies (Fig. 6). MVA pathway proteins were detected both in the supernatant and in the pellet. The Western blot analysis also showed a PHLS accumulation preferentially in the pellet, consistent with the results of Fig. 5. Interestingly, transformants with the MVA operon appeared to contain more PHLS in the supernatant compared with

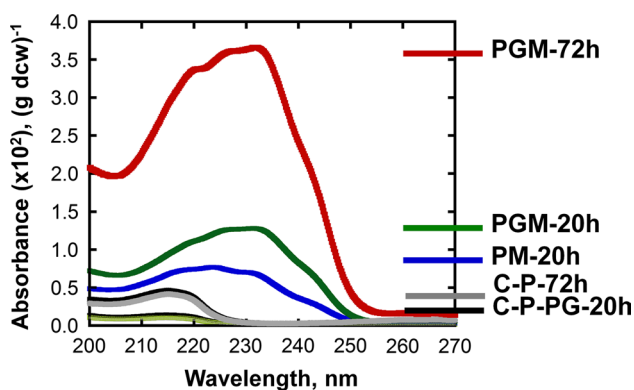


Fig. 7 Absorbance spectra of hexane extracts from *E. coli* transformants normalized on a per g dcw, following a batch incubation of the cells. *E. coli* cultures were grown in LB media supplemented with 1 % glucose. Absorbance spectra of hexane extracts from untransformed (control) *E. coli* and different transformants were normalized on g of dcw. C, Rosetta cells serving as control. P, PHLS. PG, PHLS-GPPS. PM, PHLS plus MVA pathway. PGM, PHLS-GPPS plus MVA pathway. The absorbance spectra are the average of at least three independent biological replicates. Variance of the spectral peak amplitudes was within ± 20 % of the reported value. β -Phellandrene production was assayed either after 20 or 72 h incubation of the batch cultures

the other clones (Fig. 6, PHLS). Conversely, GPPS was detected only in the pellet (Fig. 6, GPPS). The Western blot analysis confirmed expression of the heterologous proteins, and validated the assignment of the transgenic bands in the Coomassie-stained SDS-PAGE of Fig. 5.

β -Phellandrene was collected along with the hexane solvent from the surface of the cultures and analyzed by UV absorbance spectroscopy (Fig. 7). The absorbance spectra of the hexane extracts from different transformants were normalized on per g of *E. coli* dcw. Control cultures (Fig. 7, C) and those expressing the PHLS gene alone (Fig. 7, P) or in combination with the heterologous expression of the GPPS gene (Fig. 7, PG) failed to show β -phellandrene in the hexane extract. Only upon co-expression of the MVA pathway, β -phellandrene was produced and accumulated to detectable levels in the hexane extracts from transformant *E. coli* cultures (Fig. 7, PM, PGM). The corresponding absorbance spectra clearly showed a primary band peaking at 232.4 nm that matched the absorbance profile of a β -phellandrene standard (Formighieri and Melis 2014). Expression of the MVA pathway in combination with the PHLS yielded 5.7 mg β -PHL per g dcw, over a 20 h incubation period. Upon the additional heterologous expression of the GPPS, the yield was increased to 11 mg β -PHL per g dcw, over the same incubation time. It is evident that the MVA pathway provides additional carbon flux enabling β -phellandrene synthesis. The positive effect of the heterologous GPPS expression further suggests low intracellular levels of geranyl-diphosphate, which constitutes a

secondary rate- and yield-limiting step. This observation may indicate that endogenous GPPS activity in *E. coli* is still limiting under our experimental conditions.

The β -phellandrene production assay was extended to 72 h of cell incubation in the LB plus 1 % glucose media. Control and transformants with the PHLS gene alone did not produce any β -phellandrene, as evidenced by absorbance spectral analysis at 232.4 nm (Fig. 7, C–P–72 h). In the specific case of *E. coli* growth in LB media supplemented with 1 % glucose, there was a non-specific absorbance at 215 nm, present in the hexane extract from both untransformed (control) and PHLS-transformed cells (Fig. 7, C–P–72 h). This band was attributed to the accumulation of a compound unrelated to β -phellandrene, probably resulting from the glucose-driven endogenous cellular metabolism. As such, it did not interfere with the β -phellandrene absorbance at 232.4 nm or with the GC analysis of products with a 14.3 min retention time. Both absorbance spectrophotometry and GC analysis indicated that, after 72 h incubation, β -phellandrene accumulated to 25 mg β -PHL per g dcw in the culture. It is concluded that *E. coli* growth in LB media supplemented with 1 % glucose is superior to minimal media supplemented with glycerol in terms of sustaining the energetic demands of the cell, and also in terms of provision of substrate for the isoprenoid biosynthetic pathway, thereby resulting in improved β -phellandrene production.

Discussion

A chemoheterotrophic bacterial system for the production of fuel and chemicals, such as the one offered by *E. coli*, is typically based on plasmid expression of the required transgenes and, further, on provision of exogenous carbon source to sustain cell growth and productivity. This is in contrast to a photosynthetic bacterial system, such as the one offered by *Synechocystis*, which is typically based on chromosomal expression of the required transgenes with photosynthesis as the source driving cell growth and productivity. Advantage of the plasmid-based transformation is that transgenes can be expressed to levels sufficient to alleviate rate limitations due to low concentrations of the catalytic enzymes. A plasmid-based expression, however, requires the presence of a selectable marker (antibiotic) to maintain the plasmids and the associated transgenes in the cell, and this adds to the economic and environmental burden of organic carbon (glucose or glycerol), which is needed for growth and productivity (Zurbriggen et al. 2012). Advantage of the chromosomal-based photosynthetic transformation (e.g., *Synechocystis*) is that transgenes are stably incorporated, alleviating the need to use selectable markers. However, levels of expression of transgenes are generally low, potentially causing enzyme limitations

in the catalytic rate of product formation (Formighieri and Melis 2014). The latter applies to terpene hydrocarbon synthesis, as terpene synthases have a slow k_{cat} of 4–5 s⁻¹, necessitating enhanced levels of the enzyme to support high rates of product synthesis (Formighieri and Melis 2014).

Additional substantial differences exist in the regulation of the MEP pathway between the chemoheterotrophic and photosynthetic microbial systems for terpene synthesis, as the innate cellular isoprenoid to biomass ratio is substantially lower in the former than in the latter. The reason for this difference is that photosynthetic but not chemoheterotrophic organisms must synthesize relatively high amounts of carotenoids, phytol tails for chlorophyll, and plastoquinone molecules to serve the photosynthetic apparatus, all of which are not present in the chemoheterotrophic system. It is implied that endogenous carbon partitioning to the MEP isoprenoid biosynthetic pathway is much greater in the photosynthetic than in the chemoheterotrophic cell. A schematic representation of comparative isoprenoid biosynthesis in *E. coli* (Fig. 8, left) and *Synechocystis* (Fig. 8, right) depicts the much greater cellular carbon investment in *Synechocystis* than in *E. coli*. In these bacteria, IPP and DMAPP are synthesized via the endogenous MEP pathway, and serve as universal precursors for the synthesis and accumulation of terpenoid products that are essential for cellular function, such as prenyl-tRNA, prenyl-heme, prenyl-ubiquinones and menaquinones, and bactoprenol, the latter required for cell wall biosynthesis. *Synechocystis* specifically synthesizes hopanoids from squalene, and a squalene-hopene cyclase has been recently characterized (Englund et al. 2014). In contrast, no squalene or hopanoids synthesis has been reported in *E. coli* from its endogenous metabolism. In addition, photosynthetic activity in *Synechocystis* requires the synthesis and accumulation of substantial amounts of prenylated plastoquinones, phytol moieties of chlorophylls, and tetraterpene-derived carotenoids, none of which are present or needed in the *E. coli* system. It was estimated that *Synechocystis* partitions about 4–5 % of its internal carbon in the isoprenoid biosynthetic pathway to serve the above-described needs (Lindberg et al. 2010; Melis 2012). The corresponding carbon investment in *E. coli* is estimated to be much smaller, as this microorganism lacks the abundance of terpenoids required by the photosynthetic apparatus. This comparative analysis substantiates the notion that carbon partitioning and flux through the isoprenoid biosynthetic pathway is naturally modulated to suit the specific needs of the cell, and therefore, flux through the MEP pathway can be manipulated to improve yields (Melis 2013).

As a consequence of the greater cellular carbon investment of *Synechocystis* into the isoprenoid pathway, heterologous transformation of *Synechocystis* with the *PHLS* gene alone resulted in the photosynthetic production and

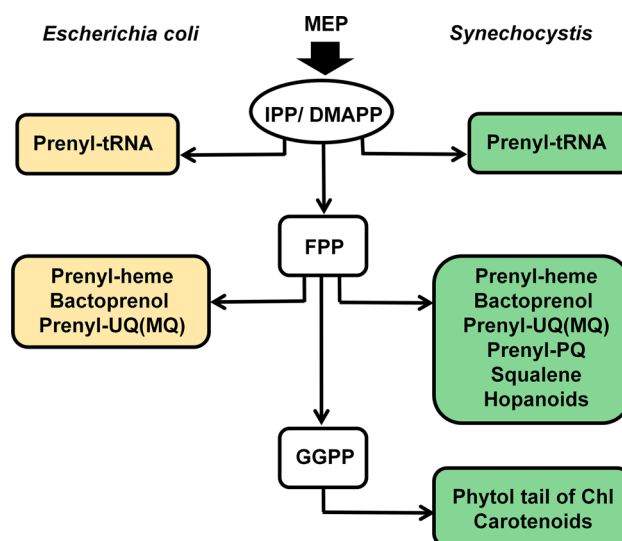


Fig. 8 Schematic representation of the endogenous isoprenoid biosynthetic pathway and its products in *E. coli* (left) and *Synechocystis* (right). IPP and DMAPP are synthesized via the endogenous MEP pathway, and serve as universal precursors for the synthesis and accumulation of terpenoids essential for cell function. In *E. coli*, these include prenyl-tRNA, prenyl-heme, prenyl-ubiquinones and menaquinones, and bactoprenol, the latter required for cell wall biosynthesis. *Synechocystis* synthesizes, in addition, hopanoids from squalene, while no squalene or hopanoids accumulation has been detected in *E. coli* from the endogenous cellular metabolism. Furthermore, the photosynthetic activity of *Synechocystis* requires the synthesis and accumulation of substantial amounts of prenylated plastoquinones, phytol moieties of chlorophylls, and carotenoids, none of which are present or needed in the *E. coli* system. As a consequence of the specific cell requirements, cellular carbon allocation to the isoprenoid pathway was estimated to be 4–5 % of the *Synechocystis* biomass, while it is far less in *E. coli*

accumulation of β -phellandrene as a floater molecule in the liquid culture (Bentley et al. 2013; Formighieri and Melis 2014). In *Synechocystis*, the limitation in β -phellandrene yield was due to low expression levels of the *PHLS* enzyme, rather than substrate availability (Formighieri and Melis 2014). In contrast, the present work showed that substrate availability is the yield-limiting factor in *E. coli* (Figs. 3, 7). Substantial amounts of β -phellandrene accumulated only when *E. coli* was heterologously transformed with the *MVA* operon along with the *PHLS* gene alone or with the *PHLS*-*GPPS* combination. These results suggested that the endogenous MEP pathway in *E. coli* might provide sufficient substrate for the few isoprenoids required for cell growth, and also for low levels of isoprene production (Zurbriggen et al. 2012), but does not have the flux capacity to generate significant amounts of monoterpene products. This limitation is clearly alleviated, when heterologous expression of the *MVA* pathway increases flux and substrate pressure in the direction of IPP and DMAPP synthesis.

Unlike plants, algae, and cyanobacteria, heterotrophic microorganisms usually do not encode a specific GPPS, and promiscuous farnesyl-diphosphate synthase (FPPS) activities include the synthesis of both GPP and FPP (Fischer et al. 2011). In the present work, we showed a significant endogenous geranyl-diphosphate synthase activity in *E. coli*, supporting the synthesis of 5.7 mg β -PHL per g dcw during 20-h incubation, provided that heterologous expression of the MVA pathway enables sufficient carbon flux for enhanced IPP and DMAPP synthesis (Figs. 3, 7, PM). These results also suggest that IPP and DMAPP availability is the primary limiting factor in β -phellandrene synthesis, rather than a low intracellular level of geranyl-diphosphate.

In conclusion, heterologous expression of the *PHLS* gene is necessary, but not sufficient to enable β -phellandrene production in *E. coli*. Isoprenoid pathway flux amplification upon heterologous expression of the MVA pathway was required for transgenic monoterpene biosynthesis in this system. Variance between *E. coli* and the cyanobacterium *Synechocystis* in the production of β -phellandrene was attributed to a substantially slower endogenous carbon flux through the MEP isoprenoid biosynthetic pathway in *E. coli* relative to that in *Synechocystis*.

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