The isoprenoid alcohol pathway, a synthetic route for isoprenoid biosynthesis

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Edited by Tobias J. Erb, Max Planck Institute for Terrestrial Microbiology (MPG), Marburg, Germany, and accepted by Editorial Board Member Caroline S. Harwood May 15, 2019 (received for review December 13, 2018)

The more than 50,000 isoprenoids found in nature are all derived from the 5-carbon diphosphates isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Natively, IPP and DMAPP are generated by the mevalonate (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways, which have been engineered to produce compounds with numerous applications. However, as these pathways are inherently constrained by carbon, energy inefficiencies, and their roles in native metabolism, engineering for isoprenoid biosynthesis at high flux, titer, and yield remains a challenge. To overcome these limitations, here we develop an alternative synthetic pathway [illegible] T1.2448TL.90200.90238.381155054(i1214m(velsubsequ.(tngi-310.2(e)-sphate3.6(

Table 1. Comparison of native and synthetic pathways for the generation of isoprenoid precursors

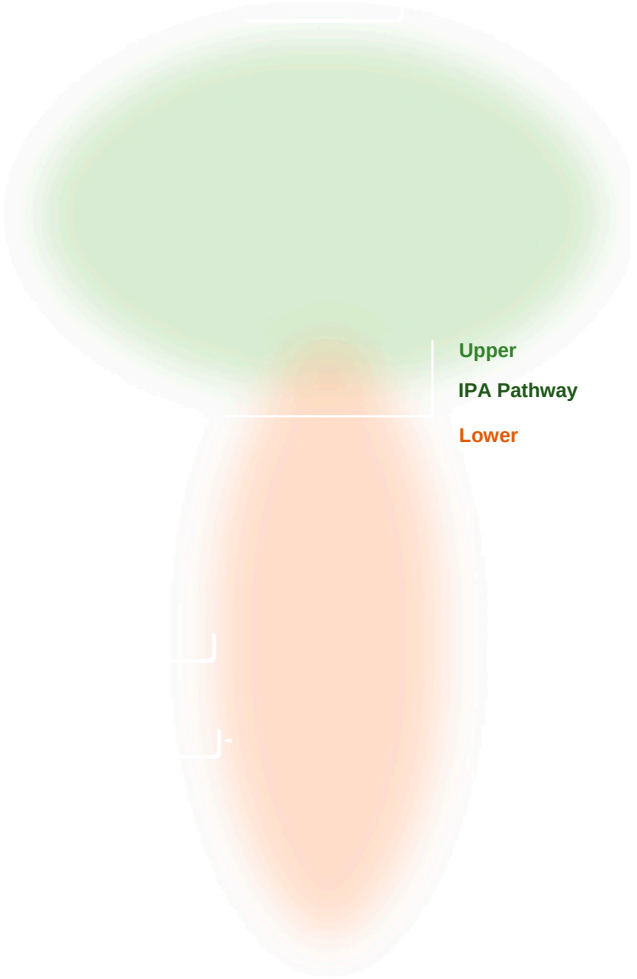
	Native isoprenoid pathways		Isoprenoid alcohol pathway		
Attribute*	MVA	MEP	Fig. 4*A*	*SI Appendix*, Fig. S10[†]	*SI Appendix*, Fig. S12[‡]
Starting central carbon metabolite(s)	Acetyl-CoA	Pyruvate; glyceraldehyde 3-phosphate	Acetyl-CoA	Acetyl-CoA; propionyl-CoA	Pyruvate
Number of steps[§]	6	7	8	8 (17)	10
Initial isoprenyl precursor produced	IPP	IPP and DMAPP	DMAPP	DMAPP (GPP)	DMAPP
Carbon efficiency[¶]	0.83	0.83	0.83	1.00	0.83
ATP balance[#]	−3 ATP	−3 ATP (1 ATP; 1 CTP to CMP)	−2 ATP	−2 (−2) ATP	−2 ATP
Redox balance[#,‖]	−2 NAD(P)H	−3 NAD(P)H	−2 NAD(P)H	−3 (	

[e.g., geranyl pyrophosphate (GPP)/C_{10}, farnesyl pyrophosphate (FPP)/C_{15}, etc.], all share a common chemical structure with an aliphatic hydrocarbon chain (C_{5n}) forming a phosphate ester with a pyrophosphate group (Fig. 1). Considering this general structure, a retrobiosynthetic analysis suggests that these isoprenoid precursors can be generated from the corresponding alcohols through the phosphorylation of the hydroxyl group followed by a second phosphorylation of the resulting phosphate esters (Fig. 1). When these sequential phosphorylation reactions are combined with pathways for the synthesis of IPAs from central carbon metabolites, a non-native route to isoprenoid biosynthesis is realized (Fig. 1). As IPAs are the key metabolic intermediates linking isoprenoid precursors to central carbon metabolites, we termed this design the IPA pathway. In this pathway architecture, an IPA(s) connects the upper IPA branch, generating C_{5n} IPA(s) from central carbon intermediates and the lower IPA branch converting the C_{5n} IPA(s) to various isoprenoid precursors (e.g., DMAPP, IPP, GPP, etc.) and, hence, facilitates the synthesis of isoprenoid compounds (Fig. 1). In principle, any C_{5n} IPA could serve as the intermediate(s), however, targeting C_5 (iso)prenol would enable the generation of all chain length pyrophosphate intermediates through the formation of DMAPP and IPP and subsequent iterative condensation with C_5 units.

Identification and Characterization of Enzymes Supporting Lower IPA Pathway Designs. Our design targeting (iso)prenol as the key intermediate(s) for generating universal isoprenoid precursors relies on the ability to phosphorylate either C_5 IPA to the corresponding pyrophosphate intermediate (Fig. 1). Although (iso)prenol is not known to be the physiological substrate of any kinase, reports have confirmed the ability for various thermophilic isopentenyl phosphate kinases (IPKs) to phosphorylate both (iso)prenol as well as the monophosphate (e.g., DMAP) (11, 12), and a protein engineering strategy was recently utilized to construct a *Thermoplasma acidophilum* IPK variant (IPK_{THA*}) with improved prenol phosphorylation activity (11). In attempting to utilize IPK_{THA*} for catalyzing (iso)prenol kinase activity, we found it to have low soluble expression in *Escherichia coli* resulting in low levels of purified IPK and hence a potential for low functional activity to limit the utility of this enzyme.

Our initial efforts to identify a better performing enzyme(s), through both sequence similarity search via HMMER (13) and mining for candidate enzyme sequences using our desired reaction(s) via Selenzyme (14), returned a vast number of potential enzymes difficult to narrow to the most promising subset (*SI Appendix*, Figs. S2 and S3). Given the low soluble expression of our template protein in *E. coli* and the relatively low level of (iso)prenol kinase activity to begin with (11, 12), parsing this set of enzymes for ideal candidates to test remains a challenge. As such, we utilized a more rational approach wherein the $–CH_x–CH_2OH$ structure of (iso)prenol was used to identify potential *E. coli* enzymes catalyzing the phosphorylation of a primary alcohol with a double bond, methyl branch, or functional group at carbon 2 or 3 (Fig. 2*A*). Of the enzymes identified, purification and characterization of prenol kinase activity revealed that *E. coli* hydroxyethylthiazole kinase (ThiM) can catalyze our desired phosphorylation reaction (Fig. 2*A*).

To assess the lower IPA pathway for conversion of (iso)prenol to C_{10} isoprenoids, we combined the expression of ThiM with the IPK from *Methanothermobacter thermautotrophicus* (IPK_{MTH}), which in addition to its native function of converting IP to IPP can also convert DMAP to DMAPP ($k_{cat}/K_m = 7.9 \times 10^5$) (12). Furthermore, *E. coli* IPP isomerase (Idi) was overexpressed for the interconversion of DMAPP and IPP, and a truncated version of geranyl diphosphate synthase (GPPS2) from *Abies grandis* (15) was utilized for subsequent condensation of DMAPP and IPP to GPP (Fig. 1). Geraniol production was selected as a proxy for GPP generation with the expression of a truncated version of the geraniol synthase (GES) from *Ocimum basilicum* enabling conversion of GPP to geraniol (16). Using a biotransformation approach in which a growth and expression phase was followed by resuspension of the cells in minimal media containing 10 mM prenol, nearly 200 mg/L geranoids (C_{10} IPAs geraniol, nerol, and citronellol) were produced when ThiM was overexpressed in conjunction with other lower IPA pathway enzymes (*SI Appendix*, Fig. S4). The substrate and product flexibility of terpene synthases (2) as well as endogenous enzymes for generating or interconverting isoprenoid products (17) are likely contributing causes to the formation of three distinct monoterpenoids observed here. Although ThiM also appears capable of phosphorylating isoprenol as the same combination of enzymes resulted in ~125 mg/L C_{10} IPAs when 10 mM isoprenol was used (*SI Appendix*, Fig. S4), the 60% higher geranoid production using prenol indicated this C_5 alcohol was preferable. Furthermore, the use of ThiM in place of the aforementioned IPK_{THA*} resulted in more than a 15-fold increase in geranoids from prenol (*SI Appendix*, Fig. S4). Characterization of *E. coli* ThiM further confirmed the ability for this enzyme to catalyze the phosphorylation



of prenol with the following kinetic parameters: $K_m = 5.8 \pm 0.1$ mM; $k_{cat} = 0.11 \pm 0.01$ s^{-1}; $k_{cat}/K_M = (1.9 \pm 0.09) \times 10^1$ M^{-1} s^{-1} (*SI Appendix*, Fig. S5).

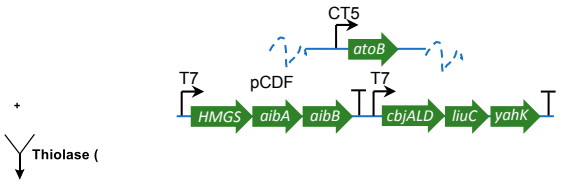
Fermentative Production of Isoprenoids through a Lower IPA Pathway. To better assess the ability of the lower IPA pathway to support product synthesis, we evaluated isoprenoid production in fermentations with cells actively growing on a common carbon source (glycerol). For this, we utilized a two-plasmid system for the overexpression of lower IPA pathway enzymes in a genetic background, MG1655 derivative JST06(DE3), engineered for the synthesis of various products from glycerol (18). The ThiM-mediated lower IPA pathway enabled geranoid production from prenol (Fig. 2*B*). Since significant amounts of prenol remained unutilized in the medium after 48 h, we hypothesized and confirmed that product/geraniol toxicity was a major issue, including its intracellular accumulation (*SI Appendix*, Fig. S6). To alleviate these issues, we utilized a 15% dodecane overlay which enabled the complete utilization of ∼2 g/L prenol and significantly reduced the accumulation of intracellular geranoids (*SI Appendix*, Fig. S6*B*) yielding a total of ∼1.5 g/L geranoids (Fig. 2*B*). When 3 g/L of prenol was included, over 2 g/L of geranoids were produced compared with only ∼0.005 g/L without kinase overexpression (Fig. 2*C*), which demonstrates minimal generation of diphosphate intermediates through the native MEP pathway.

We also explored the use of various condensation and product synthesis enzymes (Fig. 1). Replacement of GES with a truncated version of the limonene synthase from *Mentha spicata* (19) enabled the production of nearly 0.5 g/L limonene (Fig. 2*C*). We next utilized alternative enzymes for the condensation of diphosphate intermediates in conjunction with appropriate termination enzymes (20, 21) to demonstrate the synthesis of longer-chain products, farnesol, a sesquiterpenoid (C_{15}), as well as the C_{30} and C_{40} isoprenoids diaponeurosporene and lycopene, respectively (Fig. 2 *C* and *D*). Although the lower product titers for these longer-chain products could be explained, in part, due to the competition for FPP (e.g., ubiquinone and menaquinone biosynthesis) (2), the ratio of DMAPP to IPP generated via the phosphorylation of C_5 IPAs may also play a key role, especially with the use of prenol which dictates that DMAPP is the initial C_5 pyrophosphate generated. Indeed, when isoprenol, instead of prenol, was utilized as the feed for the lower IPA pathway, farnesol production was observed (Fig. 2*D*). As such, the utilization of *E. coli* Idi, which favors the formation of DMAPP over IPP (22), may limit the availability of IPP units required for longer-chain length isoprenoids. In agreement with this hypothesis, the use of an Idi from *Streptomyces* sp. strain CL190 (23), an enzyme that favors the formation of IPP over DMAPP (22, 23), led to the production of ∼40 mg/L farnesol from prenol compared with negligible amounts with the use of *E. coli* Idi (Fig. 2*D*). Interestingly, neither strategy significantly improved diaponeurosporene or lycopene production (*SI Appendix*, Fig. S8) indicating that additional constraints beyond the DMAPP to IPP ratio are potentially limiting C_{30} and C_{40} product synthesis from the IPA pathway.

Design and In Vivo Implementation of the Upper IPA Pathway. Capitalizing on the ability of the engineered lower IPA pathway to support the synthesis of isoprenoid precursors in the context of the overall synthetic route requires upper IPA pathway designs for the efficient generation of IPAs. However, to date, the synthesis of iso(prenol) has only been accomplished via cleavage of the pyrophosphate group from the corresponding isoprenoid intermediate, essentially the reverse of the IPA pathway reactions, following engineering of native isoprenoid pathways (24, 25). To generate upper IPA routes to these C_5 alcohols from C_2 and C_3 central carbon metabolites, enzymes catalyzing carbon–carbon bond forming Claisen, aldol, or acyloin condensation reactions were considered (Fig. 1). When combined with other biochemical reactions and various starting points from the central carbon metabolism, these condensation reactions could support an array of different routes to IPAs of varying chain lengths (*SI Appendix*, Figs. S9–S12). Although the design and prototyping of the lower IPA pathway indicated that prenol would be preferable as a linking metabolite, our initial designs for the upper IPA pathway also considered isoprenol and longer-chain length IPAs to determine all potential routes.

The potential and practical applications of select routes were assessed through various metrics, including both quantitative carbons, ATP, and redox balances, and qualitative factors, such as the complexity and availability of the specific biochemical reactions, in direct comparison with native isoprenoid pathways (Table 1). Although pathways based on nondecarboxylative Claisen condensation of acetyl-CoA and propionyl-CoA, and decarboxylative condensation of two molecules of pyruvate have promising carbon, ATP, and

redox balances (Table 1), implementation of these pathways would be limited, in part, due to the need for challenging biochemical reactions for which no known enzyme has demonstrated the required substrate to product conversion (



prenol synthesized from glycerol was further converted to our desired product. Further evaluation of conditions (media volume, IPTG concentration, and dodecane overlay; *SI Appendix*, Fig. S16) and the use of 40 g/L glycerol resulted in significant increases to geranoid production with nearly 0.6 g/L geranoids (0.59 ± 0.06 g/L) produced after 72 h (Fig. 4*C*) compared with 0.013 ± 0.004 g/L with the control strain without the overexpression of ThiM and IPK_{MTH} (*SI Appendix*, Fig. S17). Geraniol represented the major C_{10} product with only small amounts (<20 mg/L) of nerol and citronellol produced (Fig. 4*D*). Prenol production was also observed with increasing concentration throughout the time course (Fig. 4*C*). These results mark the demonstration of a synthetic isoprenoid pathway to produce isoprenoid compounds from a single unrelated carbon source.

Discussion

We have demonstrated the design, prototyping, and implementation of a non-natural pathway to isoprenoid biosynthesis, termed the IPA pathway (Fig. 1). The IPA pathway is based on the conversion of central carbon metabolites to C_{5n} alcohol(s) (upper IPA), which are subsequently phosphorylated to the corresponding isoprenoid precursors (lower IPA). The identification of a native *E. coli* kinase (ThiM) capable of phosphorylating (iso)prenol facilitated the development of a lower IPA pathway capable of supporting isoprenoid pyrophosphate generation and enabling the synthesis of multiple isoprenoid products, including geraniol, limonene, farnesol, diaponeurosporene, and lycopene (Fig. 2). With prenol as the linking metabolite between the upper and the lower IPA pathways, we developed a route to this branched unsaturated alcohol from central carbon metabolites recruiting biochemical reactions from various organisms that enabled its selective production at nearly 2 g/L (Fig. 3). When the upper and lower branches were combined into an integrated IPA pathway, a full synthetic route to isoprenoids from the single unrelated carbon source glycerol was realized, resulting in the production of 0.6 g/L monoterpenoids (Fig. 4).

The IPA pathway design circumvents many of the stoichiometric and regulatory constraints associated with native isoprenoid pathways, which have evolved for the generation of biosynthetic intermediates required for cell growth opposed to efficient product synthesis (2). For example, whereas the MVA and MEP pathways both consume three net ATP equivalents per 5-carbon diphosphate generated from their respective starting central carbon metabolites (3), the IPA pathway requires two ATP molecules (Table 1). The IPA pathway variant demonstrated here is relatively easy to implement as it does not require complex chemistry or enzymes with intrinsic characteristics that limit their potential in vivo functionality under various conditions. Compared with the MVA pathway, the IPA pathway avoids the tight regulation associated with many pathway enzymes, such as 3-hydroxy-3-methyl-glutaryl-CoA reductase proposed to be regulated

at the transcriptional, post-transcriptional, translational, and post-translational level, including inhibition by free CoA, HMG, NADPH, and $NADP^+$ (3, 32).

We have also identified potential bottlenecks for which future engineering efforts may serve to improve product synthesis and overall pathway operation. Specific to longer-chain length isoprenoids, control of the DMAPP to IPP ratio appears to represent an important factor, especially when prenol is the linking IPA. For overall IPA pathway operation, the accumulation of prenol in the medium indicates that the lower pathway, specifically prenol kinase, represents a key bottleneck. In addition, the development of alternative upper IPA routes to IPAs has the potential to further improve pathway operation. For example, preliminary results for the route proceeding via pyruvate through an acetolactate synthase (*SI Appendix*, Fig. S12) suggest a potential for high titer and flux (33). Finally, although our demonstration of this synthetic isoprenoid pathway was implemented in *E. coli*, accessing the vast range of isoprenoid products requires the use of other microbial systems, such as yeast(s), which offer distinct advantages for the production of high value isoprenoids and oxygenated derivatives (5). Given its modularity and design directly linking to central carbon metabolism, expression of the IPA pathway could be explored in a range of micro-organisms to further unlock the potential of this route for isoprenoid synthesis.

Materials and Methods

Strains, Plasmids, and Genetic Methods. Wild-type K12 *E. coli* strain MG1655 (34) was used as the host for all genetic modifications with gene knockouts and chromosomal expression constructs introduced by P1 phage transduction. Strains used in this study as well as details on plasmid construction and molecular biology techniques can be found in the *SI Appendix*.

Culture Medium and Cultivation Conditions. The minimal medium designed by Neidhardt et al. (35), with 125 mM MOPS and Na_2HPO_4 in place of K_2HPO_4, supplemented with 20 g/L glycerol, 10 g/L tryptone, 5 g/L yeast extract, 100 μM $FeSO_4$, 5 mM calcium pantothenate, 1.48 mM Na_2HPO_4, 5 mM $(NH_4)_2SO_4$, and 30 mM NH_4Cl was used for all fermentations unless otherwise stated. Calcium carbonate was included at 5.5% wt/vol for pH control. Fermentations were conducted in 25 mL Pyrex Erlenmeyer flasks (Corning, Inc., Corning, NY) filled with varying media volumes and foam plugs filling the necks. Individual flasks from the same inoculum were stopped at various times to determine cell growth and product synthesis profiles. Additional details, including an estimation of potential product losses due to volatilization, can be found in the *SI Appendix*.

Analytical Methods. OD was measured at 550 nm in a Thermo Spectronic Genesys 20 (Thermo Scientific, Waltham, MA) and used as an estimate of cell mass ($1\ OD_{550\ nm} = 0.34$ g dry weight/L). Concentrations of glycerol, ethanol, and organic acids were determined via HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a refractive index detector and a HPX-87H organic acid column (Bio-Rad, Hercules, CA) with the following operating conditions: 0.3 mL/min flow rate, 30 mM H_2SO_4 mobile phase, column temperature 42 °C. Quantification and identification of additional compounds were carried out using an Agilent 7890B series custom gas chromatography system equipped with a 5977B inert plus mass selective detector turbo EI bundle (for identification), a flame ionization detector (for quantification), and an Agilent HP-5ms capillary column (0.25-mm internal diameter, 0.25-μm film thickness, 30-m length) as described in the *SI Appendix*.

Enzyme Assays. Details on the preparation of cell extracts, protein purification, and specific enzyme assays are described in the *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by Bioactive Ingredients Corporation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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