Article

Transcriptional Tuning of Mevalonate Pathway Enzymes to Identify the Impact on Limonene Production in *Escherichia coli*

Jonghyeon Shin,[∥] Eric J. South,[∥] and Mary J. Dunlop*

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ABSTRACT: Heterologous production of limonene in microorganisms through the mevalonate (MVA) pathway has traditionally imposed metabolic burden and reduced cell fitness, where imbalanced stoichiometries among sequential enzymes result in the accumulation of toxic intermediates. Although prior studies have shown that changes to mRNA stability, RBS strength, and protein homology can be effective strategies for balancing enzyme levels in the MVA pathway, testing different variations of these parameters often requires distinct genetic constructs, which can exponentially increase assembly costs as pathways increase in size. Here, we developed a multi-input transcriptional circuit to regulate the MVA pathway, where four chemical inducers, L-arabinose (Ara), choline chloride (Cho), cuminic acid (Cuma), and isopropyl β-D-1-thiogalactopyranoside (IPTG), each regulate one of four orthogonal promoters. We tested modular transcriptional regulation of the MVA pathway by placing this circuit in an engineered *Escherichia coli* "marionette" strain, which enabled systematic and independent tuning of the first three enzymes (AtoB, HMGS, and HMGR) in the MVA pathway. By systematically testing combinations of chemical inducers as inputs, we investigated relationships between the expressions of different MVA pathway submodules, finding that limonene yields are sensitive to the coordinated transcriptional regulation of HMGS and HMGR.
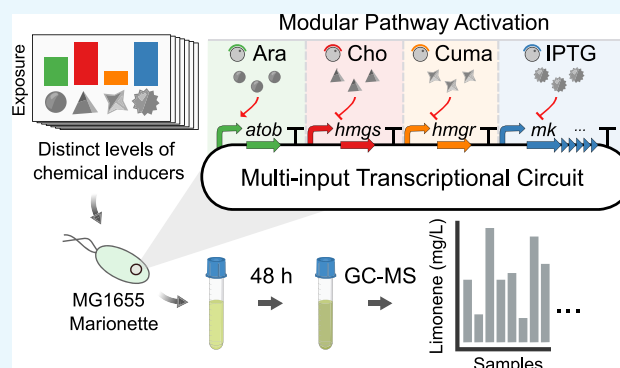
INTRODUCTION

Limonene is part of a diverse family of isoprenoids that are naturally produced in hundreds of plants and animals and in some bacteria.[1−3] Over the past few decades, limonene has been the focus of countless metabolic engineering efforts due to its wide-ranging functional roles in industry (e.g., fragrances, food additives, and biofuels). As the market for limonene consumption continues to grow,[4,5] there are incentives to better characterize the anabolic pathways involved in producing isoprenoids and to further develop bioengineering methods for inexpensive, bulk production of limonene and its derivatives in microorganisms.[6−10] Limonene is derived from the universal isoprenoid precursors IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate),[6,11−13] which can be biosynthesized from either the mevalonate synthesis (MVA) pathway or the methylerythritol 4-phosphate (MEP) pathway.[1,14,15] Depending on which downstream enzymes are used, IPP and DMAPP can be further synthesized into a variety of commodity terpenoids.[2]

Previous studies have sought to enhance titers, rates, and yields of limonene and its derivatives in microorganisms with various success.[2,7,16−19] However, these studies have commonly reported adverse interactions between the heterologous isoprenoid biosynthesis pathway and the host's central carbon metabolism.[3,16,20,21] The poorly integrated metabolic pathways

can perturb native regulatory mechanisms in a cell, impose excessive metabolic burden, and risk both reduced cell fitness and decreased product titers.[2,16,17,21−24] This is because porting heterologous, non-evolved metabolic pathways into an organism can cause imbalanced stoichiometries among sequential enzymes, which can result in both suboptimal metabolic flux and the accumulation or depletion of chemical intermediates.[25−27] Given the importance of pathway balancing, methods for precise tuning of the activity of sequential enzymes in a metabolic pathway through synthetic regulatory control have been the subject of intense study.[2,7,24,28,29]

Balancing the expression of sequential enzymes has historically involved multivariate modular metabolic engineering, an approach where metabolic pathways are split into distinct submodules and simultaneously varied.[27,30] Metabolic pathway variants, each associated with a distinct combination of genetic parts and gene expression levels, are then compared
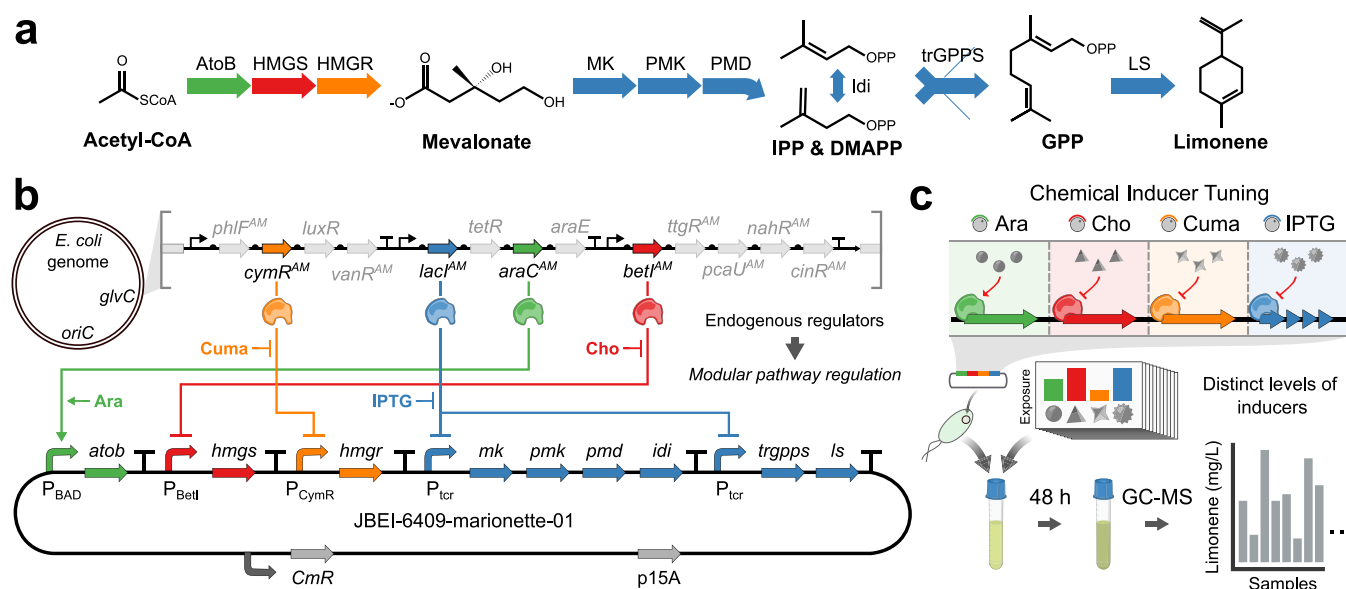
Figure 1. Modular transcriptional regulation of the mevalonate pathway to tune limonene production in *E. coli*. (a) Limonene production pathway, where acetyl-CoA serves as the starting substrate in the mevalonate (MVA) pathway, which then over multiple steps is consumed alongside NADPH and ATP to produce IPP or DMAPP and then limonene. Abbreviations: AtoB, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate pyrophosphate decarboxylase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; Idi, isopentenyl diphosphate isomerase; trGPPS, truncated geranyl diphosphate synthase; GPP, geranyl diphosphate; LS, limonene synthase. (b) Marionette *E. coli* strain MG1655, where heterologous repressors are integrated into the genome at the *glvC* locus. These endogenous repressors are constitutively expressed and will bind to their cognate promoters on a plasmid to regulate downstream gene expression. Four inducible promoters (P_{BAD}, P_{BetI}, P_{CymR}, and P_{tcr}, induced by L-arabinose (Ara), choline chloride (Cho), cuminic acid (Cuma), and isopropyl β-D-1-thiogalactopyranoside (IPTG), respectively) are inserted to modularize the limonene synthesis pathway. (c) A marionette transcriptional circuit enables both modular and multivariate application of chemical inducers to tune the expression of enzymes. Limonene production after 48 h of fermentation is measured by gas chromatography−mass spectrometry (GC−MS).

based on their observed bioproduction performance.[2,20,27,31−33] Multivariate modular metabolic engineering has enabled the systematic study of exogenous MVA pathways in cells,[2,7,16,17] where transcriptional,[26,34,35] post-transcriptional,[33] and post-translational[28] rebalancing strategies have been demonstrated for enhancing mevalonate titers. Specifically, strategies to alter the stoichiometries of MVA pathway enzymes have involved modifying intergenic regions (e.g., gene linkers and RBS sites) on operons,[20] altering the stability of mRNA transcripts,[1,33] CRISPR-based gene knockdowns,[17] and substrate channeling with synthetic protein scaffolds.[28] Moreover, *in vitro* prototyping of the MVA pathway with cell-free systems has been shown to optimize limonene production, where different combinations of homologous enzymes, protein concentrations, and reaction conditions were tested in high-throughput.[32,36,37]

Although studies have shown that nontranscriptional parameters can significantly impact MVA pathway flux,[1,20,21,33,38] testing different variations of these parameters requires distinct genetic constructs, which can exponentially increase both library sizes and assembly costs as enzymatic pathways increase in length. However, synthetic biology continues to mature and bring about tools for tuning the expression of multiple genes in parallel, which are enabling new methods to rapidly compare putative enzyme stoichiometries in metabolic pathways with a minimal set of genetic variants. Here, we take advantage of the "marionette" system in *Escherichia coli*, designed for the modular control of up to 12 genes in parallel.[29] Briefly, marionette strains enable the rapid study of enzyme rebalancing by coupling the expression of

each gene in a metabolic pathway to a particular small-molecule inducer. Adding different amounts of chemical inducers will control the activity of individual promoters and, therefore, the expression of genes in the associated module (Figure 1a,b). Since adding different combinations of small-molecule inducers into the medium is straightforward, marionette strains can be used in systematic screens to compare how different transcriptional induction profiles among genes in a metabolic pathway influence end-product formation. An initial study using this approach to optimize biosynthetic pathways showed great promise, increasing yields in the five-enzyme lycopene pathway to 90 mg/L.[29]

Here, we tested to what extent multivariate addition of chemical inducers could be leveraged as a tool to improve limonene bioproduction in *E. coli*. Using a marionette strain, we constructed a multi-input transcriptional circuit to systematically tune the transcriptional regulation of genes within the MVA pathway and then compared how changes in gene expression influenced final limonene titers.

■ RESULTS

Four-Dimensional Control of Transcriptional Regulation. *E. coli* does not naturally produce limonene, so we engineered a plasmid derived from Alonso-Gutierrez et al.,[15] encoding both the MVA pathway and necessary downstream enzymes under the control of inducible promoters, which we then transformed into an *E. coli* MG1655 marionette strain. The constructed MVA pathway is regulated by four orthogonal promoters, which enables the independent control of gene expression using the following four chemical inducers: L-
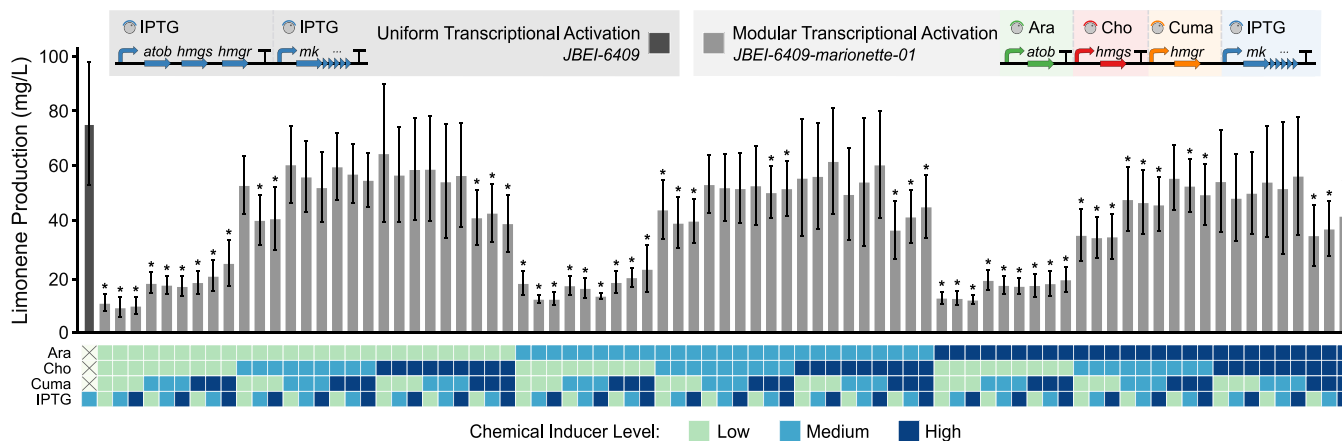
Figure 2. Limonene production in marionette *E. coli* strains across 81 chemical inducer combinations. Transcriptional regulation of different submodules of the MVA pathway significantly impacts limonene titers in batch culture after 48 h of fermentation. Error bars represent standard deviation among replicates for each combination of chemical inducers ($n = 6-8$ biological replicates). Chemical inducer levels corresponding to low, medium, and high: arabinose (2, 10, and 50 μM), choline chloride (50, 200, and 500 μM), cuminic acid (2, 5, and 20 μM), and IPTG (25, 100, and 200 μM). Strains harboring the JBEI-6409 plasmid serve as a positive control. This uniform transcriptional activation is achieved using a single inducer, where P_{lacUV5} and P_{trc} promoters were induced with a medium concentration (100 μM) of IPTG. This JBEI-6409 strain produced a 76 mg/L limonene titer. Modular multivariate transcriptional regulation was implemented with strains harboring the JBEI-6409-marionette-01 plasmid. A normalized version of this figure, which accounts for day-to-day variation between batches, is shown in Figure S1. Asterisks mark statistically significant differences in limonene titers between a given modular transcriptional activation sample (cells with JBEI-6409-marionette-01) and the uniform transcriptional activation control (cells with JBEI-6409). The statistical significance for normalized data was determined with Welch's unequal variances *t*-tests ($\alpha = 0.05$), followed by *p*-value correction for multiple testing using the Benjamini−Hochberg procedure (FDR = 0.10) (Table S3).

arabinose (Ara), choline chloride (Cho), cuminic acid (Cuma), and isopropyl β-D-1-thiogalactopyranoside (IPTG). Our marionette-based circuit primarily targets the upper MVA pathway, where the first three enzymes (AtoB, HMGS, and HMGR) are each transcriptionally regulated by a different small-molecule inducer (Figure 1b).

Similar to the original marionette study, we discretized the extent to which chemical inducers were added to samples into three levels: low (near-zero), intermediate (half-maximum), and high (near-maximum).[29] To determine the chemical concentrations that would represent these levels, we referenced dose−response curves for Ara, Cho, Cuma, and IPTG from the original marionette study, which linked inducer-specific concentrations to relative gene expression levels for cells growing in LB at the mid-log phase.[29] With this experimental setup, we performed an exhaustive grid search, where the four inducers regulated the transcriptional submodules at three different levels of gene expression. This creates a four-dimensional search space. The 81 multivariate levels of chemical inducers were each added to limonene-producing *E. coli* cultures, which were then fermented and measured using gas chromatography−mass spectrometry (GC−MS) to determine the best-balanced transcriptional regulation needed for producing high titers of limonene in the engineered host (Figure 1c).

Transcriptional Regulation of HMGS and HMGR Genes Disproportionately Influences Limonene Titers.
Cell cultures produced a wide range of limonene titers, which were dependent on how different submodules of the MVA pathway were transcriptionally regulated (Figure 2). The best- and worst-performing marionette cell cultures, each responding to a different profile of chemical inducers, displayed a 7-fold difference in limonene titers. This difference reinforces that transcriptional regulation can significantly impact the metabolic flux through the MVA pathway. Notably, cells

harboring the plasmid presented in this study (JBEI-6409-marionette-01) did not generate superior limonene titers compared to cells with the plasmid from Alonso-Gutierrez *et al.* (JBEI-6409;[15] used as the positive control), in which transcription of the entire MVA pathway was uniformly induced with IPTG to produce 76 mg/L limonene (Figure 2). Using a subset of the inducer combinations, we also verified that growth was similar for all conditions we tested and was not dependent on the limonene production level or inducer concentration (Figure S2). The fact that limonene titers were lower among all cell samples harboring JBEI-6409-marionette-01 suggests that the presented four-dimensional search space, regulating individual enzymes in the upper operon of the MVA pathway, does not produce scenarios where transcript levels facilitate rates of translation that achieve both optimal enzyme stoichiometric ratios and limonene production. Alternatively, improvements to limonene production may depend on conducting a similar transcriptional grid search with enzymes in the bottom half of the MVA pathway: MK, PMK, PMD, and Idi. However, identifying which interactions negatively impact limonene titers provides valuable insight into the key requirements for pathway balancing within the MVA pathway.

To determine which combinations of inducers were the most impactful for overall limonene production, we sorted samples (i.e., the 81 induction profiles) and binned them into distinct quartile groups based on their final limonene titers (Figure 3a). Frequencies of each pairwise combination of chemical inducers were then compared across quartile groups. Since each quartile group was associated with a distinct proficiency for limonene production, comparing which pairs of chemical inducers were enriched in a given quartile summarizes how transcriptional regulation of different MVA pathway submodules impacts the overall biosynthetic performance.
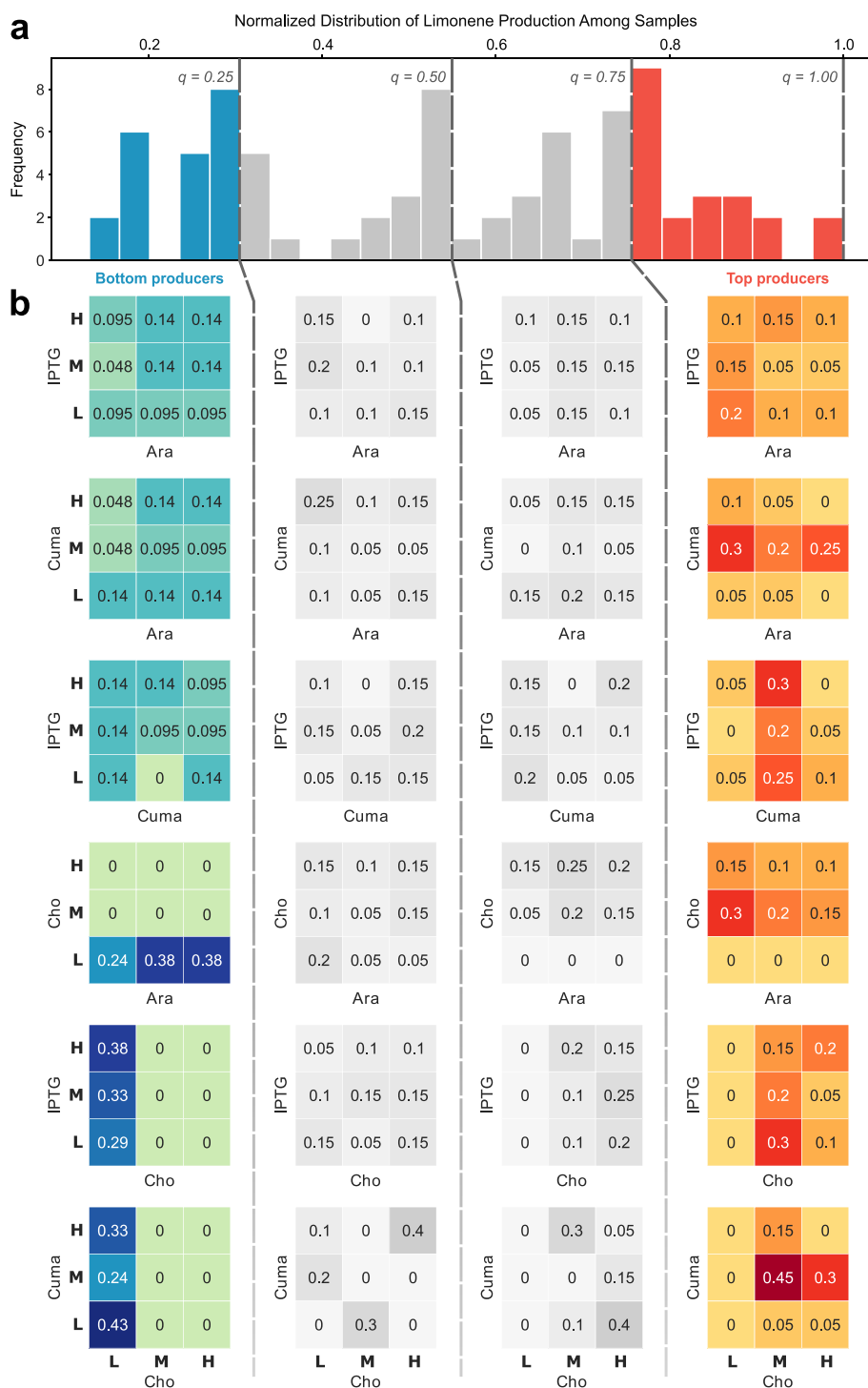
Figure 3. Pairwise relationships between MVA pathway submodules demonstrate optimal production with balanced HMGS and HMGR. (a) Samples that corresponded to 81 different chemical induction profiles are sorted into distinct quartile groups based on their normalized limonene titers. (b) Proportion of each pairwise combination of chemical inducers among quartile groups. A value of zero corresponds to no samples with this pair of inducers falling in the quartile; a value of one corresponds to all samples with this inducer falling in the quartile. Low transcriptional activation with Cho, corresponding to low HMGS expression, was enriched among the poorest limonene producers (bottom left). Medium doses of both Cho and Cuma (i.e., moderate transcriptional activation of HMGS and HMGR) were enriched among the highest limonene producers (bottom right). Darker colors correspond to higher frequency. L = low; M = medium; H = high.

Limonene yields in this study were the most sensitive to the transcriptional regulation of HMGS and HMGR. Low transcriptional regulation with Cho, corresponding to low HMGS expression, was enriched in the low limonene-producing quartile group, where a low abundance of the

enzyme became flux-limiting for the MVA pathway (Figure 3b). The top limonene producers among the 81 induction profiles were cells that received a medium dose of both Cho and Cuma (i.e., moderate transcriptional activation of HMGS and HMGR). The MVA pathway achieves optimal flux when

these bottleneck enzymes reside in an expression window between too low (flux-limiting) and too high (cytotoxic). This result corresponds well with our positive control, JBEI-6409, where both genes were regulated by P_{lacUV5}, which has been characterized as a medium-strength promoter.[39] In contrast, no degree of Ara or IPTG dosages was enriched among quartiles, suggesting that the strength of P_{BAD} and P_{tcr} was strong enough, even under low chemical induction, to express a sufficient amount of enzyme to adequately process intermediates and maintain MVA pathway flux.

The association between optimal limonene production and moderate expression of HMGS and HMGR may be explained by how the heterologous MVA pathway interacts with its host's physiology. Low levels of HMGS can result in the redirection of carbon flux toward acetate as opposed to mevalonate, and high levels of HMGR can disrupt the intracellular redox balance.[40] At the system level, acetyl-CoA serves as the starting substrate in the MVA pathway, which then over multiple steps is consumed alongside NADPH and ATP to produce IPP or DMAPP.[20,22,32] However, acetyl-coA is part of the tricarboxylic acid (TCA) cycle and plays a vital role in other aspects of primary metabolism. Therefore, cells must maintain sufficient acetyl-coA pools to feed both the needs of any engineered MVA pathway alongside other native functions.[2,7] In addition, HMG-CoA (the intermediate metabolite produced by HMGS and used as a substrate by HMGR) has been shown to inhibit fatty acid biosynthesis and reduce both MVA pathway productivity and cell viability.[2,21] Indeed, multiple enzymes and cofactors within the MVA pathway have been previously characterized or associated with cytotoxic effects. Overall, we found that changes in transcriptional regulation of HMGS and HMGR were the main determinant of final limonene titers in cell culture.

◼ DISCUSSION

Here, we tested to what extent limonene biosynthesis could be influenced by transcriptional rebalancing of the MVA pathway in *E. coli*. Using a marionette strain, we constructed a multi-input transcriptional circuit to systematically tune the expression of four biosynthetic submodules, which together comprised a complete route toward limonene biosynthesis. We found that output limonene titers were the most sensitive to alterations in transcriptional regulation of the HGMR and HMGS genes in our experimental setup. The trends presented in this study corroborate results from another study by Alonso-Gutierrez *et al.*,[38] where limonene production and protein levels were compared while expressing the nine mevalonate pathway enzymes across different scenarios. Utilizing both targeted proteomics and modular metabolic engineering, the balanced expression of enzymes, on a plasmid similar to JBEI-6409, was found to be more productive than the over-expression of a single gene. Furthermore, high levels of HMGS and HMGR were associated with low limonene production.[38] In another corroborating work, Dueber *et al.* used synthetic protein scaffolds to compare mevalonate production titers to the stoichiometry of HMGS and HGMR, which led to the observation that medium abundances of these enzymes resulted in the highest mevalonate titers in culture.[28] Notably, through use of the marionette system in this study, we compared how putative changes in enzyme levels both balanced the MVA pathway and improved limonene production without the need for development of multiple genetic constructs or the use of protein scaffolds.

Modifications to HMGS and HMGR, whether by replacing these genes in *E. coli* with orthologs from another bacterium (*Staphylococcus aureus*) or using truncated protein variants, have also been demonstrated to increase mevalonate titers.[6] As a result, expanding the list of either known orthologs or beneficial point mutations among HMGS or HMGR proteins would help future attempts to improve MVA pathway flux.[6] Finally, beyond HMGS and HMGR, MK and Idi have also been hailed as "bottleneck" enzymes in previous studies,[1,2,8,12,17,20,28] suggesting that future engineering efforts to carefully tune their expression levels could improve yields.

While the multivariate, modular tuning of the MVA pathway may help optimize the production of limonene in cells, more innate issues such as inefficient enzymes, cross-reactivity with native metabolisms, and non-optimal intracellular conditions remain grand challenges when designing microbes for isoprenoid biosynthesis.[2] It is worth noting that the marionette system, although capable of rapidly testing various expression profiles, is not capable of testing enzyme homologs in rapid succession, unlike cell-free systems.[37] Future efforts to optimize the MVA pathway may benefit from using both the marionette system and cell-free pipelines in parallel or from incorporating the marionette system with targeted proteomics to verify whether multivariate transcriptional regulation can generate a wide range of protein stoichiometries *in vivo*.[37,38] Furthermore, it would also be interesting to perform reverse transcription real-time PCR (RT-qPCR) experiments while conducting multivariate screens with the marionette system, which could further decipher how different transcription levels for each gene in the MVA pathway correspond to final limonene titers.

This work adds to the growing list of strategies for modulating the expression of enzymes in the MVA pathway for improved isoprenoid biosynthesis in *E. coli*. Looking ahead, temporal control of enzyme expression could be considered when using the marionette system, as "just-in-time" strategies for the transcription of enzymes have been shown to improve the productivity of metabolic pathways.[42,43] Optimization efforts in this growing combinatorial space can benefit from emerging data-driven multiplexed techniques for pathway design to make these complex screens more manageable.[37,44−46] It is worth noting that other organisms, such as the yeast *Yarrowia lipolytica*, have also been used to produce limonene,[41] and it would be interesting to see how similar multivariate transcriptional tuning of the MVA pathway, using a system analogous to the marionette system, would translate in eukaryotic cells.

◼ MATERIALS AND METHODS

Strains, Media, Chemical Inducers, and Plasmids. The strain used in this work was derived from the *E. coli* "marionette-wild" MG1655.[29] We removed the *cat* gene from the genome using a pCP20 plasmid encoding the *flp* gene. Cells were grown in either LB (Miller, BD Difco, 244610) or M9 minimal media composed of M9 minimal salts (BD Difco, 248510; 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, and 0.5 g/L NaCl), 1% D-glucose (Sigma-Aldrich, G5767), 0.2% casamino acids (Fisher Bioreagents, BP1424-500), 0.34 g/L thiamine hydrochloride (Fisher Bioreagents, 04700-100), 2 mM $MgSO_4$ (Fisher Chemical, M87-100), and 0.1 mM $CaCl_2$ (Fisher Chemical, C79-500). Chloramphenicol (30 mg/L; Acros Organics, 227920250) was used to select and maintain plasmids. Chemical inducers used as inputs were

arabinose (Acros Organics, 104981000), choline chloride (Sigma-Aldrich, 102226316), cuminic acid (Sigma-Aldrich, 1002950587), and IPTG (Fisher Bioreagents, BP1755-10).

The plasmid controlled by the marionette strain was derived from JBEI-6409 (used as a positive control), which encodes enzymes for limonene synthesis.[15] From this JBEI-6409 plasmid, the *lacI* gene was removed, and arabinose-, choline chloride-, and cuminic acid-inducible promoters were inserted to control the *atob*, *hmgs*, and *hmgr* genes, respectively. We denote this plasmid JBEI-6409-marionette-01. All plasmid modifications were completed using the Gibson assembly cloning method.[47] Plasmids are listed in Table S1, and Note S1 provides sequences.

Growth and Induction of Limonene Production Strains. Cells harboring either limonene production plasmid (JBEI-6409 or JBEI-6409-marionette-01) were streaked on an LB plate supplemented with chloramphenicol. Single colonies were picked, inoculated, and grown overnight in LB with chloramphenicol at 37 °C at 200 rpm (New Brunswick, Excella E25). Cells were then back-diluted to an OD_{600} of 0.10 in 5 mL of M9 media with chloramphenicol and then grown at 37 °C at 200 rpm. When cells reached an OD_{600} between 0.80 and 1, appropriate concentrations of inducers (arabinose: low (2 μM), medium (10 μM), and high (50 μM); choline chloride: low (50 μM), medium (200 μM), and high (500 μM); cuminic acid: low (2 μM), medium (5 μM), and high (20 μM); IPTG: low (25 μM), medium (100 μM), and high (200 μM)) were added in each culture, and 20% dodecane (e.g., 1.0 mL of dodecane to 5.0 mL total volume) was layered on top of the liquid culture. Induced cells were grown at 30 °C at 250 rpm for 48 h. Limonene production among samples was then measured by GC−MS. The dodecane layer above each cell culture was transferred into microcentrifuge tubes and centrifuged at 25,000g for 1 min. Fifty microliters of the sample-derived dodecane and limonene mixture was diluted with 450 μL of ethyl acetate containing 10 mg/L α-pinene (Acros Organics, 131261000) as an internal standard for the quantification in a 2 mL glass vial (Agilent Technologies, 5182-0716; 5185-5820).

Limonene Quantification with GC−MS and Postprocessing. Limonene samples were analyzed with an Agilent GC−MS 6890N equipped with an MS detector for up to 800 m/z. Helium was used as a carrier gas at a constant flow rate of 1 mL/min in an Agilent 222-5532LTM column. The inlet temperature was set to 300 °C. The oven temperature was held at 50 °C for 30 s, ramped up to 150 °C at a rate of 25 °C/min, and then further ramped to 250 °C at a rate of 40 °C/min. The results were analyzed using the MSD Productivity ChemStation (E.02.02.1431). This software returns area percentages for each peak in an output chromatogram. Since the area of a peak is proportional to the amount of a compound in a sample, area percentages for α-pinene (internal standard; 10 mg/L) in each chromatogram (i.e., each sample) were used as a reference to calculate limonene concentrations from limonene area percentages.

The 81 cell cultures, each with distinct chemical induction profiles, were processed over the span of 2 weeks. To mitigate experimental batch effects between days, limonene titers were normalized using the positive control (strains harboring JBEI-6409) for each corresponding day. Overall, multiple normalized limonene titers were generated for each sample ($n = 6-8$ replicates), which were then aggregated to produce a final normalized average (see Table S2).

■ ASSOCIATED CONTENT

⑤ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00483.

> Figure S1: normalized limonene production in marionette *E. coli* strains; Figure S2: growth under different inducer concentrations; Table S1: plasmids used in this study; Note S1: DNA sequences used in this study (PDF)

> Table S2: limonene production titers as a function of inducer level for all replicates; Table S3: statistical testing results (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

Mary J. Dunlop − *Biomedical Engineering Department and Molecular Biology, Cell Biology & Biochemistry Program, Boston University, Boston, Massachusetts 02215, United States;* ⦿ orcid.org/0000-0002-9261-8216; Email: mjdunlop@bu.edu

Authors

Jonghyeon Shin − *Biomedical Engineering Department, Boston University, Boston, Massachusetts 02215, United States;* Present Address: Present address: Manus Bio, 1030 Massachusetts Ave. #300, Cambridge, Massachusetts 02138, United States (J.S.)

Eric J. South − *Molecular Biology, Cell Biology & Biochemistry Program, Boston University, Boston, Massachusetts 02215, United States;* ⦿ orcid.org/0000-0003-4426-3729

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00483

Author Contributions

∥J.S. and E.J.S. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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