Distributing a metabolic pathway among a microbial consortium enhances production of natural products

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Metabolic engineering of microorganisms such as Escherichia coli and Saccharomyces cerevisiae to produce high-value natural metabolites is often done through functional reconstitution of long metabolic pathways. Problems arise when parts of pathways require specialized environments or compartments for optimal function. Here we solve this problem through co-culture of engineered organisms, each of which contains the part of the pathway that it is best suited to hosting. In one example, we divided the synthetic pathway for the acetylated diol paclitaxel precursor into two modules, expressed in either S. cerevisiae or E. coli, neither of which can produce the paclitaxel precursor on its own. Stable co-culture in the same bioreactor was achieved by designing a mutualistic relationship between the two species in which a metabolic intermediate produced by E. coli was used and functionalized by yeast. This synthetic consortium produced 33 mg/L oxygenated taxanes, including a monoacetylated dioxygenated taxane. The same method was also used to produce tanshinone precursors and functionalized sesquiterpenes.

Plants synthesize numerous structurally complex compounds that have important therapeutic properties, for example, paclitaxel (Abraxane), a potent antitumor agent. Heterologous production of these molecules in industrial microbes—mainly bacteria and yeasts—could provide a robust and sustainable production process. However, in bacteria it has been challenging to functionally express sophisticated eukaryotic enzymes that are often required in the synthesis of complex compounds; on the other hand, it has been equally difficult to engineer yeasts for high-yield production of building blocks of natural products, such as the isoprenoid biosynthetic pathway of bacteria, which has higher theoretical yield than that of yeasts.

In nature, microbes can form interacting communities to accomplish chemically difficult tasks through division of labor among different species. These natural microbial consortia have been used in food and other industries for decades. Furthermore, researchers studied interactions of microbial species in mixed microbial cultures extensively in the 1960s and 1970s, aiming to establish operating diagrams for maintaining synthetic co-culture, which has been challenging owing to differences in their doubling time and secretion of toxic metabolites. Recently, there have been reports of production of biofuels and chemicals by a few synthetic consortia comprising genetically engineered microbes. However, those studies were mostly concerned with the stability of microbial consortia, whereas the more recent work focused on using nonconventional biomass such as cellulose. In these examples, which each involved two species, the first species provided only the carbon source for the second, which harbored the essential pathway for the final product in its entirety and was able to make the final product on its own. Strictly speaking, none of this prior work examined the potential to use more than one species for the purpose of constructing a long synthetic pathway, which would enable production of structurally complex compounds.

In this study, we demonstrate the concept of reconstituting a heterologous metabolic pathway in a microbial partnership in which one microbe is engineered to synthesize a metabolic intermediate that is translocated to another microbe, in which it is further functionalized. In principle, it could be attractive to use synthetic microbial consortia for production of valuable metabolites, especially those with complex structures. One major advantage of this design is that each expression system and pathway module can be constructed and optimized in parallel, so that the time required for making the product would be substantially reduced. Other advantages of using synthetic consortia include, (i) taking advantage of unique properties and functions of different microbes, (ii) exploring beneficial interactions among consortium members to enhance productivity and (iii) minimizing problems arising from feedback inhibition by means of spatial pathway module segregation.

We report the use of two model laboratory and industrial microbes, E. coli and S. cerevisiae, in a consortium to produce precursors of the anti-cancer drug paclitaxel. E. coli is a fast-growing bacterium that can be engineered to overproduce taxadiene, the scaffold molecule of paclitaxel. S. cerevisiae, having advanced protein expression machinery and abundant intracellular membranes, has been suggested as a preferable host for expressing cytochrome P450s (CYPs), which functionalize taxadiene by catalyzing multiple oxygenation reactions. We find that integration of parts of the whole pathway in separate species cultured together combines dual properties of rapid production of taxadiene in E. coli with efficient oxygenation of taxadiene by S. cerevisiae. This approach has overcome the challenges of using E. coli alone, in which fine-tuned taxadiene production was perturbed by introducing CYPs and functional expression of these enzymes in E. coli.
RESULTS

Co-culture design to produce paclitaxel precursors

We first engineered *S. cerevisiae* BY4700 to express a cytochrome P450 taxadiene 5α-hydroxylase (5αCYP) and its reductase (CPR) (5αCYP-CPR, fused as a single polypeptide; Supplementary Fig. 1a), which catalyze the first oxygenation reaction in the pathway of paclitaxel biosynthesis. Taxadiene was efficiently oxygenated by this yeast (named TaxS1) when taxadiene was externally fed into the culture medium (Supplementary Fig. 1b), confirming that the 5αCYP was functional in *S. cerevisiae* BY4700. Next, we co-cultured this 5αCYP-CPR–expressing yeast with a taxadiene-producing *E. coli* (named TaxE1) in a fed-batch bioreactor with glucose as the sole carbon and energy source (Fig. 1a). The mixed culture produced 2 mg/L of oxygenated taxanes in 72 h (Fig. 1b), whereas in control experiments in which only *E. coli* TaxE1 (Fig. 1b) or *S. cerevisiae* TaxS1 (data not shown) was cultured, no oxygenated taxanes were produced. These results showed that taxadiene produced by *E. coli* diffused into *S. cerevisiae* and was subsequently oxygenated. However, the cell density of *E. coli* (Fig. 1c) and the total titer of taxanes (Fig. 1d) were substantially reduced in the presence of *S. cerevisiae* compared to the absence of yeast. The cause could be inhibition of *E. coli* by accumulated ethanol produced by yeast when grown on glucose (Fig. 1e). This hypothesis was validated by the fact that ethanol, at the highest concentration observed (50 g/L, Fig. 1e), completely inhibited *E. coli* cell growth and taxadiene production (Supplementary Fig. 2). Similar instances of inhibition have been observed before in natural systems when microbes compete for common resources (18).

To overcome this problem, we designed a mutualistic interaction between the two microorganisms (18). When *E. coli* metabolizes xylose it excretes acetate, which is inhibitory to its own growth (19). *S. cerevisiae*, on the other hand, cannot metabolize xylose but can use acetate as the sole carbon source for growth without producing ethanol (Fig. 2a and Supplementary Table 1). We therefore switched the co-culture carbon source from glucose to xylose. Under these conditions, *S. cerevisiae* grew in the xylose medium only in the presence of *E. coli* (Fig. 2b), and the concentration of extracellular acetate in the co-culture was considerably reduced compared with that observed when *E. coli* was grown on xylose on its own (Fig. 2c). More importantly, this stable co-culture minimized the ethanol concentration to below the limit of detection (0.1 g/L) throughout the experiment. In addition, the titer of total taxanes produced by *E. coli* was not substantially affected by the presence of *S. cerevisiae* (Fig. 2d), suggesting that ethanol inhibition of *E. coli* was successfully eliminated, and taxadiene production proceeded unabated by the presence of yeast. However, although more oxygenated taxanes were produced in this co-culture (4 mg/L in 72 h, Fig. 2e) compared with the previous co-culture (2 mg/L in 72 h, Fig. 1b), the taxadiene oxygenation efficiency was still low (only 8% of total taxadiene produced, Fig. 2).

Optimization to improve taxadiene oxygenation

To increase taxadiene oxygenation, we first focused on optimizing the growth of *S. cerevisiae*, using the rationale that more yeast cells would express more 5αCYP and therefore functionally more taxadiene. We noted that acetate accumulated in the co-culture during the first 24 h (Fig. 2c), indicating that the initial yeast population was insufficient to convert all available substrate in the medium. This was corrected by increasing the initial inoculum of yeast and also periodically feeding additional carbon (xylose), nitrogen (ammonium) and phosphorous (phosphate) sources to ensure that these major nutrients were not limiting yeast growth. After these modifications, no acetate was detected throughout the entire fermentation, and the oxygenated taxane titer was improved about threefold (16 mg/L in 90 h, Fig. 3a). Under these conditions, as growth of *S. cerevisiae* was strictly limited by the amount of acetate secreted by *E. coli*, further increase of the relative amount of yeast in the culture relied on engineering the acetate pathway in *E. coli* (see below). We opted not to feed exogenous acetate in order to preserve the autonomous nature of the co-culture (Supplementary Fig. 3).

We next improved the specific oxygenation activity of yeast TaxS1. 5αCYP-CPR (Supplementary Fig. 1a) was previously expressed under a strong constitutive promoter (TEFp in the co-culture above). We replaced TEFp by GPDp (a widely used strong promoter (20)), UAS-GPDp (an enhanced version of GPDp (21)) and ACSp (promoter of acetyl-coa synthetase, a promoter from the acetate assimilation pathway (22, 23) that we hypothesized to be strong in our study as yeast TaxS1 grew on acetate) and tested taxadiene oxygenation efficiency by the corresponding strains. To this end, yeast strains (TaxS1, TaxS2, TaxS3 and TaxS4) were cultured without *E. coli* and the oxygenation rate of exogenously supplied taxadiene was measured (Fig. 3b). Based on the results of this assay, UAS-GPDp was selected as the
strongest promoter. Yeast strain TaxS4 was then co-cultured with *E. coli* TaxE1 using xylose as a substrate, and this co-culture produced significantly ($P < 0.01$) higher concentrations of oxygenated taxanes (25 mg/L in 90 h) compared with a co-culture in which the TEFp promoter was used (16 mg/L in 90 h, Fig. 3c). GPDp and ACSp were also tested in co-culture (Fig. 3c), and the results were fairly consistent with those of the feeding experiments (Fig. 3b), for example, ACSp, the promoter characterized to be weaker than TEFp in the feeding experiment, also led to lower production of oxygenated taxanes compared with TEFp in co-culture (Fig. 3c).

After increasing oxygenation efficiency in yeast, we engineered *E. coli* to overproduce acetate and thereby further potentially improve the growth rate of *S. cerevisiae* by increasing the concentration of available substrate. Production of acetate by *E. coli* is autoregulated; when acetate accumulates, *E. coli* growth is inhibited, resulting in a lower acetate production rate. First, we overexpressed the genes in the *E. coli* acetate production pathway (phosphate acetyltransferase, *pta*, and acetate kinase, *ackA*), but this increased neither the acetate production nor the oxygenation efficiency substantially (Supplementary Fig. 4). To overcome this problem, we inactivated oxidative phosphorylation by knocking out *atpFH* (346 mg/L in 120 h), and the percentage of the taxadiene oxygenated was significantly ($P < 0.01$) increased (up to 75%, Fig. 4e). Another strategy that could be tested in the future to further improve acetate production is the knockout of *E. coli* ACS, which assimilates extra-cellular acetate under certain conditions. Such a knockout might make more of the produced acetate available to the yeast strain.

Co-culture to produce monoacetylated dioxygenated taxane

We further engineered the co-culture to produce more complex paclitaxel precursors. A prevailing theory of paclitaxel early-synthesis suggests taxadien-5α-ol is acetylated at its C-5α position, followed by oxygenation at the C-10β position (Fig. 5a). Because of the modular nature of our microbial consortium, the ability to functionalize taxadien-5α-ol could be achieved by engineering of only the yeast module. Taxadien-5α-ol acetyl-transferase (TAT29) and taxane 10β-hydroxylase (10βCYP26, fused with a CYP reductase1) were co-expressed in yeast TaxS4. When the resulting yeast (named TaxS6) was co-cultured with *E. coli* TaxE4, the co-culture produced a monoacetylated dioxygenated taxane (molecular weight 346), which was identified as a single peak on the extracted ion chromatography (346 m/z, gas chromatography–mass spectrometry (GC-MS)) and was

Figure 3 Optimizing the yeast growth and engineering the yeast promoters improved production of the oxygenated taxanes. (a) Growth optimization (increasing the yeast inoculum and feeding additional nutrients) improved production of the oxygenated taxanes by more than twofold. (b) A stronger promoter (UAS-GPDp), compared to the previously used TEFp, was found by promoter screening. (c) The co-culture using UAS-GPDp also produced significantly ($P < 0.01$, based on Student’s t-test) more oxygenated taxanes than that using TEFp. Error bars, mean ± s.e.m. in all graphs (some error bars are smaller than the plot symbols). All replicates have also been plotted in all graphs (open circles). (For most points in a and c, there are two replicates for each time point; for points in b, there are at least three replicates.)
Figure 4 Inactivating oxidative phosphorylation in *E. coli* improved yeast growth and production of oxygenated taxanes. (a) Inactivation of oxidative phosphorylation forces the production of acetate, which then becomes the major pathway generating ATP in the *E. coli*. (b) The acetate-overproducing *E. coli* (TaxE4) improved for yeast growth in the co-culture. Control: TaxE1-TaxS4 co-culture; knockout: TaxE4-TaxS4 co-culture. (c) Taxadiene oxygenation efficiency was greatly improved when the *S. cerevisiae* was co-cultured with the acetate-overproducing *E. coli*. Oxygenation efficiency of the TaxE1-TaxS4 co-culture was ~50% (20 mg/L oxygenated taxanes per 40 mg/L total taxanes), and that of the TaxE4-TaxS4 co-culture was ~75% (30 mg/L oxygenated taxanes per 40 mg/L total taxanes). Error bars, mean ± s.e.m. in all graphs (some error bars are smaller than the plot symbols). All replicates have also been plotted in all graphs (open circles, except b, in which N = 4). (For most points in c, there are two replicates for each time point; for b, there are at least three replicates.)

absent from the control co-culture not expressing the TAT and 10βCYP (Fig. 5b). A 13C labeling experiment confirmed that the oxygenated diol was indeed derived from taxadiene (Supplementary Fig. 5). The identified compound could be taxadien-5α-acetate-10β-ol, an important intermediate in the paclitaxel synthesis21, because its spectrum contained many of its fragment ions (346, 303, 286, 271 and 243 m/z27, Supplementary Fig. 5). To improve the titer and yield of this compound, we used a stronger promoter for expressing TAT (strain TaxS7), and the change of promoter improved the titer from 0.6 mg/L to 1 mg/L (Fig. 5c), confirming the hypothesis that this step was limiting. We then operated the bioreactor under a xylose-limited condition, which further increased the titer and also substantially improved the yield, by reducing the xylose consumption (from ~120 g/L to 80 g/L. Fig. 5c and Supplementary Fig. 6). To our knowledge, this is the first report of a monoacetylated, dioxygenated taxane being produced from a simple substrate (xylose) in microbes, and it reveals the usefulness of the modularity of a microbial partnership for synthesis of complex metabolites.

Production of other oxygenated isoprenoids by co-culture

The *E. coli*–*S. cerevisiae* co-culture developed in this study could be used for production of any compound if one of the pathway precursors can cross cell membranes. The method should be applicable to most isoprenoids, the largest class of natural products, because their scaffold molecules can generally permeate membranes. To test this hypothesis, we examined the synthesis of another diterpene, ferruginol, the precursor of tanshinone, which is in clinical trials for treating heart disease28,29. We replaced taxadiene synthase in *E. coli* TaxE4 with two enzymes (KSL and CPS, resulting in strain TaxE7) that are required for synthesizing miltiradiene22, a membrane-crossing molecule. At the same time, in *S. cerevisiae* BY4700, we overexpressed a specific CYP and its reductase (SmCYP and SmCPR, resulting in strain TaxS8), which were reported to oxygenate miltiradiene into ferruginol28 (Fig. 6a). When *E. coli* TaxE7 and yeast TaxS8 were co-cultured in the xylose medium, the co-culture successfully produced 18 mg/L ferruginol (Fig. 6b), which exceeds the highest titer reported in the literature (10 mg/L by *S. cerevisiae*28). This shows that the co-culture concept is generally applicable to diterpenes, and demonstrates the advantages of co-culture over monoculture, that is, being able to construct parts of the pathway in parallel and achieve higher titers owing to microbial cooperation.

Finally, we synthesized a sesquiterpene—nootkatone, which is a high-end fragrance molecule30. Similarly, we replaced the taxadiene synthase and geranylgeranyl diphasphate synthase in *E. coli* TaxE4 with a sesquiterpene synthase (VALC, resulting in strain TaxE8) to produce valencene, and in yeast BY4700, we overexpressed a specific CYP and its reductase (HmCYP and AtCPR, resulting in strain TaxS9) that can oxygenate valencene30 (Fig. 6a). When these strains (TaxE8 and TaxS9) were co-cultured, they produced 30 mg/L nootkatol and a small quantity of nootkatone (0.8 mg/L, Fig. 6c). Recently,
**DISCUSSION**

Our main motivation for using a stable co-culture was the introduction of modularity to the design of pathways for microbial metabolite production by assigning a different part of the metabolic pathway to each member of a partnership or synthetic consortium. In such an experimental set-up pathway modules can be separately optimized and assembled to enable optimal functioning of the complete pathway. The examples in this report demonstrate this modularity. The screening of a better promoter for CYP expression in yeast could be carried out independent of E. coli (Fig. 3), and producing the acetylated diol in the co-culture also required modification of only one of its modules (Fig. 5).

Such modularity should substantially expedite the reconstruction of long biosynthetic pathways in microorganisms, as the construction of such pathways involves two enzymes (ISPG and ISPH) in the taxadiene biosynthetic pathway containing iron-sulfur clusters that are sensitive to ROS. Spatial segregation, in different microbes, of the pathways of taxadiene production from its oxygenation pathway prevents inactivation of ISPG/ISPH by ROS generated by CYPs.

Because of modularity of a co-culture approach, we were able to exploit advantages of the different species. Before this study, taxadiene could be overproduced only in E. coli, whereas most biochemical characteristics of the taxadiene-functionalizing enzymes were carried out in S. cerevisiae. By using E. coli to synthesize taxadiene and S. cerevisiae to functionailize it, we combined the advantages of the two species for taxane production (fast growth of E. coli and complete protein expression system of S. cerevisiae). Using co-culture, we were able to synthesize a complex taxane (putative taxadiene-5α-acetate-10β-ol) (Fig. 5) that has never been produced by microorganisms growing on a simple carbon source, and to achieve higher titers of isoprenoid production than has been reported previously (Fig. 6b).

As most synthetic microbial consortia are competitive and exploit advantages of the different species, we were able to synthesize a complex taxane (putative taxadiene-5α-acetate-10β-ol) (Fig. 5) that has never been produced by microorganisms growing on a simple carbon source, and to achieve higher titers of isoprenoid production than has been reported previously (Fig. 6b).

We applied additional genetic and growth constraints to enforce this cooperation, for instance, the respiration-deficient E. coli was forced to produce acetate as this was its primary way to generate cellular ATP (Fig. 4a), and the yeast also had to consume acetate because it cannot utilize xylose (Fig. 2a). Under such interdependency, the inoculum ratio of our co-culture can be simply set to overinoculation of yeast (the inoculum ratio of yeast to E. coli was ~40:1). This eliminated the inhibitory acetate levels but did not result in yeast overpopulation because yeast growth was strictly limited by the concentration of acetate produced by E. coli, leading to a balanced ratio of the two species (the ratio of yeast to E. coli was 1:2 at 41 h, Fig. 4b).

Furthermore, this ratio was controllable through altering the specific acetate productivity (Fig. 4b). Because of this ability to alter the consortium composition by increasing the relative yeast population, we managed to minimize accumulation of the pathway intermediate (taxadiene) and increase the titer of oxygenated taxanes (Fig. 4c and Supplementary Fig. 9).
In addition to the mutualistic design, we also explored other strategies to avoid microbial competition. The first was a two-stage culture, in which E. coli was cultured separately for a few days before mixing with an active S. cerevisiae culture. This approach allowed both microbes to grow in their preferred conditions and taxadiene to be efficiently oxygenated (Supplementary Fig. 10). However, this process required a longer cultivation time (180 h) and, additionally, it was more complicated than that of the mutualistic co-culture. We also explored a two-carbon-source strategy, in which xylose can be used only by E. coli, and ethanol (manually added at low concentration, <2 g/L) was exclusively used by yeast (Supplementary Fig. 11). A stable co-culture could be maintained under these conditions by controlling the ethanol addition, and oxygenated taxanes were also produced at a relatively high titer (8 mg/L in 130 h, Supplementary Fig. 12). However, both E. coli and S. cerevisiae produced acetate under this scheme, leading to microbial inhibition (Supplementary Fig. 12), which was eliminated in the mutualistic design.

The co-culture concept is not restricted to pairing E. coli with S. cerevisiae. We have briefly explored the use of two different E. coli strains for production of oxygenated taxanes (Supplementary Fig. 13), which worked, although the titer was low, mainly owing to lack of the mutualistic interactions present in the E. coli–S. cerevisiae co-culture. As a general guideline, a target pathway should be divided into modules, each of which should be assigned to a specific host strain so that the combined genetic traits of the consortium strains are favorable for pathway completion. These microorganisms should rely on each other for supply of an essential nutrient or detoxification of an inhibitory substance, ensuring a stable and controllable microbial composition.

A necessary condition for co-culture is that the pathway intermediates can cross cell membranes and is secreted to the extracellular medium. This property was first confirmed for taxadiene in prior studies where organic solvent mixed with E. coli cell culture was found to efficiently extract taxadiene (C20) from the cells in a bioreactor. We also measured distribution of taxadiene in E. coli, medium and yeast in this study, which confirmed that taxadiene can cross cell membranes efficiently even in the absence of an organic solvent (Supplementary Fig. 14). This physiochemical property is shared by many isoprenoids ranging from C5 to C40, including isoprene, limonene, amorphadiene and canthaxanthin. Hence, the co-culture concept should be generally applicable to the production of most isoprenoids (in this study, we have experimentally validated production of sesquiterpene and diterpene, Fig. 6).

The experiments reported here provide evidence that a secondary metabolite pathway can be reconstructed in a microbial consortium, paving the way for engineering the microbial synthesis of natural compounds with complex structures that currently cannot be efficiently synthesized in a single microbe such as alkaloids and flavonoids (including >10,000 molecules), which all derived from aromatic amino acids that can be produced in high titer, excreted by E. coli and functionalized by S. cerevisiae. The co-culture can also benefit producing short-chain dicarboxylic acids (C6–C10), whose precursors are short-chain fatty acids that can be easily produced in engineered E. coli and efficiently oxidized in the yeast expressing CYP42.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We acknowledge useful discussions and input from A. Ghaderi, F. Lam, H. Zhang, J. Avalos and W. Wang. This work was supported by National Institutes of Health grant 1-R01-GM083323-01A1 and the Singapore MIT Alliance.

AUTHOR CONTRIBUTIONS

K.Z. and G.S. conceived the project, K.Z., K.Q., S.E. and G.S. designed the experiments, analyzed the results and wrote the manuscript. K.Z., K.Q. and S.E. did all the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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1. Aijkumara, P.K. et al. Isoprenoid pathway optimization for Taxol precursor overproduction in Escherichia coli. Science 330, 70–74 (2010).
2. Padlong, C.J. et al. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496, 528–532 (2013).
3. Alonso-Gutierrez, J. et al. Metabolic engineering of Escherichia coli for limonene and perillyl alcohol production. Metab. Eng. 15, 33–41 (2013).
4. Aijkumara, P.K. et al. Terpenoid opportunities for biosynthesis of natural product drugs using engineered microorganisms. Mol. Pharm. 5, 167–190 (2008).
5. Hefferson, K. Plant-derived pharmaceuticals for the developing world. Biotechnol. J. 8, 1193–1202 (2013).
6. Meink, S. & Stager, E. Green factories for biopharmaceuticals. Curr. Med. Chem. 20, 1038–1046 (2013).
7. Chang, M.C., Euchus, R.A., Trieu, W., Ros, D.K. & Keasling, J.D. Engineering Escherichia coli for production of functionalized terpenoids using plant P450s. Nat. Chem. Biol. 3, 274–277 (2007).
8. Ogapsak, C.M., Boyle, P.M. & Silver, P.A. Natural strategies for the spatial optimization of metabolism in synthetic biology. Nat. Chem. Biol. 8, 527–535 (2012).
9. Smid, E.J. & Lacroix, C. Microbe-microbe interactions in mixed culture food fermentations. Curr. Opin. Biotechnol. 24, 148–154 (2013).
10. Fredrickson, A.G. & Stephanopoulos, G. Microbial competition. Science 213, 972–979 (1981).
11. Davison, B.H. & Stephanopoulos, G. Effect of pH oscillations on a competing mixed culture. Biotechnol. Bioeng. 29, 1127–1137 (1986).
12. Bayer, T.S. et al. Synthesis of methyl halides from biomass using engineered microbes. J. Am. Chem. Soc. 131, 6508–6515 (2009).
13. Minty, J.J. et al. Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. Proc. Natl. Acad. Sci. USA 110, 14592–14597 (2013).
14. Xia, T., Eiteman, M.A. & Altman, E. Simultaneous utilization of glucose, xylose and arabinose in the presence of acetate by a consortium of Escherichia coli strains. Microb. Cell Fact. 11, 77 (2012).
15. Guerra-Bubb, J., Croteau, R. & Williams, R.M. The early stages of taxol biosynthesis: an interim report on the synthesis and identification of early pathway metabolites. Nat. Prod. Rep. 29, 683–696 (2012).
16. Jennren, S., Long, R.M., Williams, R.M. & Croteau, R. Cytochrome P450 taxadiene 5-salpa-hydroxylase, a mechanistically unusual monoxygenase catalyzing the first oxygenation step of taxol biosynthesis. Chem. Biol. 11, 379–387 (2004).
17. Hefer, J. et al. Cytochrome P450-catalyzed hydroxylation of taxa-4(5)-11(12)-diene to taxa-4(20),11(12)-di-en-Salpa-ol: the first oxygenation step in taxol biosynthesis. Chem. Biol. 3, 479–489 (1996).
18. Nowak, M.A. Five rules for the evolution of cooperation. Science 314, 1560–1563 (2006).
19. Everman, M.A. & Altman, E. Overcoming acetate in Escherichia coli recombinant protein fermentations. Trends Biotechnol. 24, 530–536 (2006).
20. Sun, J. et al. Cloning and characterization of a panel of constitutive promoters for applications in pathway engineering in Saccharomyces cerevisiae. Biotechnol. Bioeng. 109, 2082–2092 (2012).
21. Blazek, J.; Gang, R.; Reed, B. & Alper, H.S. Controlling promoter strength and regulation in Saccharomyces cerevisiae using synthetic hybrid promoters. Biotechnol. Bioeng. 109, 2884–2895 (2012).
22. De Vincílio, G. et al. Cloning and disruption of a gene required for growth on acetate but not on ethanol: the acetyl-CoA-Coenzyme A synthetase gene of Saccharomyces cerevisiae. Yeast 8, 1043–1051 (1992).
23. Kratzer, S. & Schuller, H.J. Transcriptional control of the yeast acetyl-CoA synthetase gene, AC21, by the positive regulators CAR8 and AOR1 and the pleiotropic repressor UME6. Mol. Microbiol. 26, 631–641 (1997).
24. Cousey, T.B., Zhou, S., Shamamgum, K.T. & Ingram, L.O. Engineering the metabolism of Escherichia coli W3110 for the conversion of sugar to redox-neutral and oxidized products: homoaçetate production. Proc. Natl. Acad. Sci. USA 100, 825–832 (2003).
25. Walker, K., Schoendoern, A. & Croteau, R. Molecular cloning of a taxa-4(20),11(12)-di-en-Salpa-O-acetyl transferase cDNA from Taxus and functional expression in Escherichia coli. Arch. Biochem. Biophys. 374, 371–380 (2000).
26. Schoendorf, A., Rithner, C.D., Williams, R.M. & Croteau, R.B. Molecular cloning of a cytochrome P450 taxane 10 beta-hydroxylase cDNA from Taxus and functional expression in yeast. Proc. Natl. Acad. Sci. USA 98, 1501–1506 (2001).

27. Wheeler, A.L. et al. Taxol biosynthesis: differential transformations of taxadien-5 alpha-ol and its acetate ester by cytochrome P450 hydroxylases from Taxus suspension cells. Arch. Biochem. Biophys. 390, 265–278 (2001).

28. Guo, J. et al. CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. Proc. Natl. Acad. Sci. USA 110, 12108–12113 (2013).

29. Zhou, Y.J. et al. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J. Am. Chem. Soc. 134, 3234–3241 (2012).

30. Hom, E.F. & Murray, A.W. Niche engineering demonstrates a latent capacity for fungal-algal mutualism. Science 345, 94–98 (2014).

31. Pillai, V.C., Snyder, R.O., Gumaste, U., Thakkumkara, T.J. & Mehvar, R. Effects of transient overexpression or knockdown of cytochrome P450 reductase on reactive oxygen species generation and hypoxia reoxygenation injury in liver cells. Clin. Exp. Pharmacol. Physiol. 38, 846–853 (2011).

32. Reed, J.R., Cawley, G.F. & Backes, W.L. Inhibition of cytochrome P450 1A2-mediated metabolism and production of reactive oxygen species by heme oxygenase-1 in rat liver microsomes. Drug Metab. Lett. 5, 6–16 (2011).

33. Arbabzadeh, V.F. et al. Influence of oxidative and nitrosative stress on accumulation of diphosphate intermediates of the non-mevalonate pathway of isoprenoid biosynthesis in corynebacteria and mycobacteria. Biochemistry 77, 362–371 (2012).

34. Rontein, D. et al. CYP725A4 from yew catalyzes complex structural rearrangement of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. J. Biol. Chem. 283, 6067–6075 (2008).

35. Xue, J. & Ahring, B.K. Enhancing isoprene production by genetic modification of the 1-deoxy-xylulose-5-phosphate pathway in Bacillus subtilis. Appl. Environ. Microbiol. 77, 2399–2405 (2011).

36. Zhou, K., Zou, R., Zhang, C., Stephanopoulos, G. & Too, H.P. Optimization of amorphaadiene synthesis in Bacillus subtilis via transcriptional, translational, and media modulation. Biotechnol. Bioeng. 110, 2556–2561 (2013).

37. Doshi, R., Nguyen, T. & Chang, G. Transporter-mediated biofuel secretion. Proc. Natl. Acad. Sci. USA 110, 7642–7647 (2013).

38. Santos, C.N., Xiao, W. & Stephanopoulos, G. Rational, combinatorial, and genomic approaches for engineering l-tyrosine production in Escherichia coli. Proc. Natl. Acad. Sci. USA 109, 13538–13543 (2012).

39. Minami, H. et al. Microbial production of plant benzylisoquinoline alkaloids. Proc. Natl. Acad. Sci. USA 105, 7393–7398 (2008).

40. Choi, Y.J. & Lee, S.Y. Microbial production of short-chain alkanes. Nature 502, 571–574 (2013).

41. Leber, C. & Da Silva, N.A. Engineering of Saccharomyces cerevisiae for the synthesis of short chain fatty acids. Biotechnol. Bioeng. 111, 347–358 (2014).

42. Craft, D.L., Madduri, K.M., Eshoo, M. & Wilson, C.R. Identification and characterization of the CYP52 family of Candida tropicalis ATCC 20336, important for the conversion of fatty acids and alkanes to alpha,omega-dicarboxylic acids. Appl. Environ. Microbiol. 69, 5983–5991 (2003).
**ONLINE METHODS**

**E. coli** strains. *E. coli* TaxE1 was previously constructed by Chin Giaw Lim (Manus Bio, Cambridge, Massachusetts, USA) in our laboratory. In brief, the MEP operon (1-des-idi-isopF controlled by T7 promoter) and the TG operon (1-tgGpps controlled by T7 promoter) were integrated into locus araA and locus lacy of *E. coli* MG1655_AreaA_AendA_DE3 (ref. 1), respectively. Strains used in this study are summarized in Supplementary Table 2.

To engineer *E. coli* TaxE1 to overproduce acetate, we overexpressed pta or pta-ackA operon using a pSC101-based plasmid containing trc promoter (p5trc). pta or ackA amplified from *E. coli* MG1655 chromosome was assembled with part of p5trc by using the recently developed cross-lapping in vitro assembly (CLIVA) method43 (primer P1–P6 used), yielding plasmid p5trc-pta and p5trc-ackA, respectively. Primers used in this study are summarized in Supplementary Table 3. All the plasmids constructed in this study were validated by means of sequencing. Plasmid p5trc-pta was transformed into *E. coli* TaxE1, yielding *E. coli* TaxE2. ackA with trc promoter and terminator was amplified from p5trc-ackA and cloned into p5trc-pta using CLIVA (primer P7–P10 used), yielding plasmid p5trc-pta-trc-ackA. This plasmid was used to transform *E. coli* TaxE1, yielding *E. coli* TaxE3. After overexpression of pta and pta-ackA, we inactivated oxidative phosphorylation of *E. coli* TaxE1 by knocking out atpFH as described previously24 (primer P11 and P12 used), yielding *E. coli* TaxE4.

To co-transform *E. coli* to produce miltiradiene, we knocked out atpFH of *E. coli* TaxE5 (a strain previously constructed by Chin Giaw Lim in our laboratory, as described previously24) (primers P11 and P12 used), resulting in strain TaxE6.

Then we transformed *E. coli* TaxE6 with plasmid p5T7-KSL-CP5-GGPPS, resulting in strain TaxE7. To obtain plasmid p5T7-KSL-CP5-GGPPS, we assembled KSL and CP5, amplified from synthetic DNA, with part of p5TTG from CLIVA (primers P13–P18 used). To construct *E. coli* to produce valencene, ispA amplified from *E. coli* genome and valC amplified from synthetic DNA were assembled with part of p5TTG from *CLIVA* (primers P18–P23 used), yielding plasmid p5T7-ISP-VALC, which was used to transform into *E. coli* TaxE6, resulting in strain TaxE8.

To engineer *E. coli* to express taxadiene 5a-hydroxylase with its reductase (5aCYP-CPR, as a fusion protein), we transformed *E. coli* with plasmid p5trc-5aCYP-CPR MG1655_AreaA_AendA_DE3, yielding *E. coli* TaxE9. Plasmid p5trc-5aCYP-CPR was previously constructed by Chin Giaw Lim in our laboratory (unpublished works). To obtain this plasmid, we cloned the coding sequence of 5aCYP-CPR, amplified from p10A12T5rsoH1-tTCP1, into p5trc. To be compatible with *E. coli* TaxE9, we used *E. coli* EDE3ch1TcrMEPp5TTG1 (named as TaxE10 in this study) to produce taxadiene in the *E. coli* co-culture, as both strains were resistant to spectinomycin. An *E. coli* carrying unbalanced taxadiene synthetic pathway was also constructed in this study. We transformed *E. coli* with TaxE4 plasmid p5TT7G, resulting in strain TaxE11.

**S. cerevisiae** strains. *S. cerevisiae* BY4700 (ATCC 200886, MATa trplΔ63 ura3Δ0) was used to express the 5aCYP-CPR. Its coding sequence amplified from plasmid p10A12T5rsoH1-tTCP1 was cloned into plasmid p416-TEF (ATCC 87368) by using the restriction enzyme cloning (Xbal and HindIII, primers P24 and P25 used), yielding plasmid p416-TEFp-5aCYP-CPR. The auxotrophic marker and expression cassette of the new plasmid (URA-TEFp-5aCYP-CPR-CYCt) was cloned via CLIVA (primer P42–P46 used), yielding p426-TEFp-TAT-ACTt. The coding sequence of *Taxus cuspidata* (10β-CYP) was synthesized (as gblocks gene fragments, Integrated DNA Technologies) and cloned into pUC-PDC6-TRP via CLIVA (primer P58–P63 used), yielding pUC-PYRPC15-URA-UAGDp-10β-CYP-CPR-CYCt. The expression cassettes of these two plasmids (TEFp-TAT-ACTt and p414-TEFp-TRP) were assembled with part of the integration vector pUC-PDC6-TRP via CLIVA (primer P64–P69 used), yielding pUC-PDC6-TRP-(GPDp-10β-CYP-CPR-CYCt)-(TEFp-TAT-ACTt).

To add upstream activation sequence (UAS) to GPDp, the UAS TEF-UASCIT1 was synthesized (as gblock gene fragment, Integrated DNA Technologies) and cloned into pUC-PYRPC15-URA-GPDp-5aCYP-CPR-CYCt via CLIVA (primer P42–P45 used), yielding pUC-PYRPC15-URA-US-GPDp-5aCYP-CPR-CYCt. This plasmid was linearized by using NotI and used to transform BY4700 (YPRC15 locus), yielding yeast TaxS4. Sequences of all the synthetic genes used in this study are summarized in Supplementary Table 4.

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Characterization of the yeast cultures by feeding taxadiene. All S. cerevisiae strains were characterized in the absence of E. coli before the co-culture experiment. We used 14-ml glass tubes (Pyrex) for this type of characterization. A colony of S. cerevisiae was inoculated into 1 ml YPD medium (10 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and grown at 30 °C/250 r.p.m. until cell density OD600 reached 2. Then 10 µL of 6 g/L synthetic taxadiene stock solution (in DMSO) was added to start the experiments, and the cultures were incubated at 22°C/250 r.p.m. The same procedure was used to compare yeast growth and activity when grown on glucose or acetate, except the medium was the one used in bioreactor experiments with indicated carbon source.

Bioreactor experiments for the E. coli–S. cerevisiae co-culture. A 1-liter Bioflo bioreactor (New Brunswick) was used for all the bioreactor works in this study. In initial experiments, seed cultures of E. coli and S. cerevisiae were inoculated into 500 ml of defined medium (13.3 g/L KH2PO4, 4 g/L (NH4)2HPO4, 1.7 g/L citric acid, 0.0084 g/L EDTA, 0.0025 g/L CoCl2, 0.015 g/L MnCl2, 0.0155 g/L CuCl2, 0.003 g/L H3BO3, 0.0225 g/L Na3MoO4, 0.008 g/L Zn(CH3COO)2, 0.06 g/L Fe(III) citrate, 0.0045 g/L thiamine, 1.3 g/L MgSO4·H2O) containing 5 g/L yeast extract and 20 g/L glucose (or 20 g/L xylose). To prepare a seed culture of E. coli, we inoculated a colony of the E. coli into Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH = 7) and grown at 27 °C/250 r.p.m. overnight. 5 ml of the grown cell suspension (OD of ~6) was inoculated into the bioreactor. To prepare a seed culture of S. cerevisiae, a colony of the S. cerevisiae was inoculated into YPD medium and grown at 30 °C/250 r.p.m. until cell density OD600 reached 20. Then 10 ml of the grown cell suspension was centrifuged at 3,000 rpm for 2 min, and pellets were resuspended in PBS and inoculated into the bioreactor. In the control experiments, only E. coli or S. cerevisiae was inoculated into the bioreactor. To improve growth of the microbes (refer to Fig. 3a), ammonium phosphate was co-fed with xylose (1 g/L (NH4)2HPO4 per 5 g xylose) and more seed culture of S. cerevisiae was inoculated (pellets of 50 ml of grown cell suspension, OD600 = 20).

During the fermentation, oxygen was supplied by filtered air at 0.5 liter/min and agitation was adjusted to maintain dissolved oxygen levels at 30% (280–800 r.p.m.). The pH of the culture was controlled at 7.0 using 10% NaOH and 0.5 HCl. The temperature of the culture in the bioreactor was controlled at 30 °C until the dissolved oxygen level dropped below 40%. The temperature of the bioreactor was then reduced to 22 °C and the E. coli was induced with 0.1 mM IPTG. During the course of the fermentation, the concentration of glucose (or xylose), acetate and ethanol was monitored at constant time intervals. As the glucose concentration dropped below 20 g/L, 20 g/L of glucose was introduced into the bioreactor. As the xylose concentration dropped below 10 g/L, 50 g/L of xylose was introduced into the bioreactor.

Bioreactor experiments for the E. coli–E. coli co-culture. A half-liter of rich medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, 5 g/L K2HPO4, 8 g/L glycerol, pH7) containing 50 mg/mL spectinomycin was inoculated with 5 ml of grown culture (OD of 4) of E. coli TaxE5 and 5 ml of grown culture (OD of 4) of E. coli TaxE6.

During the fermentation, oxygen was supplied by filtered air at 0.5 liter/min and agitation was adjusted to maintain dissolved oxygen levels at 30% (280–800 r.p.m.). The pH of the culture was controlled at 7.0 using 10% NaOH. The temperature of the culture in the bioreactor was controlled at 30 °C until the dissolved oxygen level dropped below 40%. The temperature of the bioreactor was then reduced to 22 °C and the E. coli was induced with 0.1 mM IPTG. During the course of the fermentation, the concentration of glycerol and acetate was monitored at constant time intervals. Glycerol was fed into the bioreactor at the rate of 0.65 g/h.

Test tube experiments for characterizing acetate production of E. coli. A colony of E. coli was inoculated into LB medium, and incubated at 37 °C/250 r.p.m. overnight. 10 µL of grown cells were inoculated into the same medium as the one used in E. coli–S. cerevisiae bioreactors. The cell suspension was incubated at 22°C/250 r.p.m. for 96 h and samples were taken for extracellular acetate measurement.

Quantification of isoprenoids. At indicated time points, 200 µL of cell suspension was sampled and mixed with 200 µL ethyl acetate and 100 µL 0.5-mm glass beads. The mixture was vortexed at room temperature for 20 min, and clarified by centrifugation at 18,000g for 2 min. 1 µL of the ethyl acetate phase was analyzed by GC–MS (Varian Saturn 3800 GC, 2002) attached to a Varian 2000 MS. The samples were injected into a HP-5ms column (Agilent Technologies USA). Helium at flow rate 1.0 ml/min was used as the carrier gas. The oven temperature was kept at 100 °C for 1 min, then increased to 175 °C at an increment of 15 °C/min, then increased to 220 °C at an increment of 4 °C/min, then increased to 290 °C at an increment of 50 °C/min and finally held at this temperature for 1 min. The injector and transfer line temperatures were both set at 250 °C. The MS was operated under scan mode (40–400 m/z) and total ion count of taxanes was used for the quantification. Taxadiene, nootkatol and nootkatone were quantified by using the calibration curve (total ion count vs. concentration) constructed with authentic standard.

The SrCYP was reported to produce multiple oxygenated taxanes in S. cerevisiae44. After analyzing co-culture samples, we also observed many peaks on total ion chromatography (40–400 m/z, GC-MS) between 11–18.5 min, where we did not observe any peak when a sample of the single cultures was analyzed (Supplementary Fig. 16a). Five of the major peaks contained considerable amounts of 288 m/z signal (characteristic mass of mono-oxygenated taxane, 272 (taxadiene) + 16 (oxygen), Supplementary Fig. 16b). Among them, two were previously identified as oxa-cyclotaxane (OCT) and taxadien-5z-ol44 (Supplementary Fig. 17), but the other three taxanes have not been identified before (Supplementary Fig. 18). As a conservative estimate, we only considered these five oxygenated taxanes for calculating titer of the total oxygenated taxanes. As standards of these five mono-oxygenated taxanes, the monoacetylated dioxygenated taxane and ferruginol were not available, they were quantified by using the taxadiene calibration curve.

Quantification of extracellular metabolites. At the indicated time points, 1.1 ml of cell suspension was sampled and centrifuged at 18,000g for 1 min. The supernatant was sterilized by using 0.2 µm filter. 1ml of filtered supernatant was analyzed by high-performance liquid chromatography (Waters 2695 separation module coupled to Waters 410 differential refractometer) to measure concentration of extracellular glucose, xylose, acetate and ethanol. Bio-Rad HPX-87H column was used and 14 mM sulfuric acid was used as mobile phase at the flow rate of 0.7 ml/min.

Quantification of E. coli and S. cerevisiae cell number. To measure cell number of E. coli in the E. coli–S. cerevisiae co-cultures, 2 µl of cell suspension was diluted in 200 µl sterile PBS, and 2 µl of the diluted cell suspension was further diluted in 200 µl sterile PBS. 50 µl of the repeatedly diluted cell suspension was plated on LB agar plate (1.5% agar) and incubated at 37 °C for 20 h. After the incubation, only E. coli colonies were visible on the plate (the yeast colonies were only visible after at least 48 h at this condition). This method of measuring colony forming units was time consuming and low throughput. We later developed a sucrose gradient centrifugation method to quantify the cell number of both E. coli and S. cerevisiae in the co-culture. At indicated time points, 0.5 ml of cell suspension was sampled and loaded onto 1 ml of 45% sucrose solution in a 14-ml Falcon tube, which was then centrifuged at 2,100g for 2 min. Microbes in the supernatant were exclusively E. coli and those in the pellets were mostly S. cerevisiae (Supplementary Fig. 19). After this separation, the cell number of the two microbes could be quantified by measuring optical density at 600 nm.

43. Zou, R., Zhou, K., Stephanopoulos, G. & Too, H.P. Combinatorial engineering of 1-deoxy-o-xylulose 5-phosphate pathway using cross-lapping in vitro assembly (CLIVA) method. PLoS ONE 8, e79557 (2013).
44. Flagfeldt, D.B., Siewers, V., Huang, L. & Nielsen, J. Characterization of chromosomal integration sites for heterologous gene expression in Saccharomyces cerevisiae. Yeast 26, 545–551 (2009).
45. Avalos, J.L., Fink, G.R. & Stephanopoulos, G. Compartimentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat. Biotechnol. 31, 335–341 (2013).