ACS SyntheticBiology

Review

pubs.acs.org/synthbio

From Fields to Fuels: Recent Advances in the Microbial Production of Biofuels

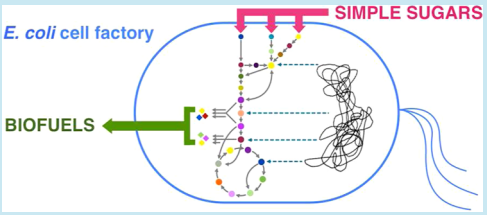
Yan Kung,[†,‡] Weerawat Runguphan,[†,‡] and Jay D. Keasling*[,†,‡,§]

[†]Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, California 94608, United States

[‡]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

[§]Departments of Chemical and Biomolecular Engineering and Bioengineering, University of California, Berkeley, Berkeley, California 94720, United States

ABSTRACT: Amid grave concerns over global climate change and with increasingly strained access to fossil fuels, the synthetic biology community has stepped up to the challenge of developing microbial platforms for the production of advanced biofuels. The adoption of gasoline, diesel, and jet fuel alternatives derived from microbial sources has the potential to significantly limit net greenhouse gas emissions. In this effort, great strides have been made in recent years toward the engineering of microorganisms to produce transportation fuels derived from alcohol, fatty acid, and isoprenoid biosynthesis. We provide an overview of the biosynthetic pathways devised in the strain development of biofuel-producing microorganisms. We also highlight many of the commonly used and newly devised engineering strategies that have been employed to identify and overcome pathway bottlenecks and problems of toxicity to maximize production titers.



KEYWORDS: *metabolic engineering, biofuels, alcohols, fatty acids, isoprenoids*

Unsustainable worldwide energy demands and the acceleration of global climate change have produced a formidable scientific research problem. Automotive gasoline, diesel, and jet fuels derived from alternative, renewable sources such as biofuels can inject a cleaner fuel into the existing transportation infrastructure currently dominated by the burning of fossil fuels. Here, synthetic biology has played a central role in the development of new microbial platforms for biofuel production (Figure 1).

Biofuels are non-fossil fuels produced from solar energy that is chemically stored as high-energy organic compounds by organisms. Atmospheric CO_2 is first fixed into sugars by photosynthesis. The sugars can then be transformed into fuel compounds by engineered microorganisms, and burning of these biofuels drives an engine, re-releasing CO_2 back into the atmosphere. This closed CO_2 cycle is driven by the energy of the sun and constitutes a more carbon-neutral process than the direct burning of fossil fuels, the cause of ever-increasing CO_2 levels.

Today, the first-generation biofuel ethanol, derived from microbial fermentation of starch from feedstocks such as corn kernels, is present in over 90% of U.S. gasoline pumps, at up to 10% concentrations.[1] However, not only do the physicochemical properties of ethanol (high hygroscopicity, low energy density, and high vapor pressure) make it ill suited as a long-term biofuel solution for our existing transportation infrastructure, the use of corn as a feedstock competes with food supply and land usage and requires vast quantities of water, fertilizers, and pesticides that themselves pose environmental hazards. Instead, a suite of biofuel compounds, including short- and medium-chain alcohols, fatty acids, and isoprenoids, is more desirable and may be produced from cellulosic biomass.[2,3] These compounds embody the forefront of advanced biofuel targets and are compatible with current gasoline, diesel, and jet fuel compositions.

Research into methods of deconstructing lignocellulosic biomass feedstocks into simple sugars in addition to the development of alternative carbon and energy sources beyond sugars are highly important and active areas of study but are not the subject of this Review. Instead, we consider recent advances in the development of microbial strains that convert simple sugars into advanced biofuel compounds, focusing on fuel types derived from alcohol, fatty acid, and isoprenoid biosynthesis. We then present an overview of both commonly utilized and novel engineering strategies that may be employed to enhance product titers, including methods of relieving metabolic bottlenecks, avoiding toxicity of intermediates and products, and overcoming pathway inhibition. Finally, we offer glimpses into some promising future directions in the development of biofuel-producing microbial strains.

FUEL TYPES

Alcohol Fuels. As mentioned above, bioethanol is a first-generation biofuel that currently dominates the biofuel infrastructure; however, other short-chain alcohols are more desirable in the long term. Recently, there have been dramatic advances in alternative alcohol production that exploit both

Special Issue: Synthetic Biology for Strain Development

Received: August 10, 2012
Published: November 5, 2012

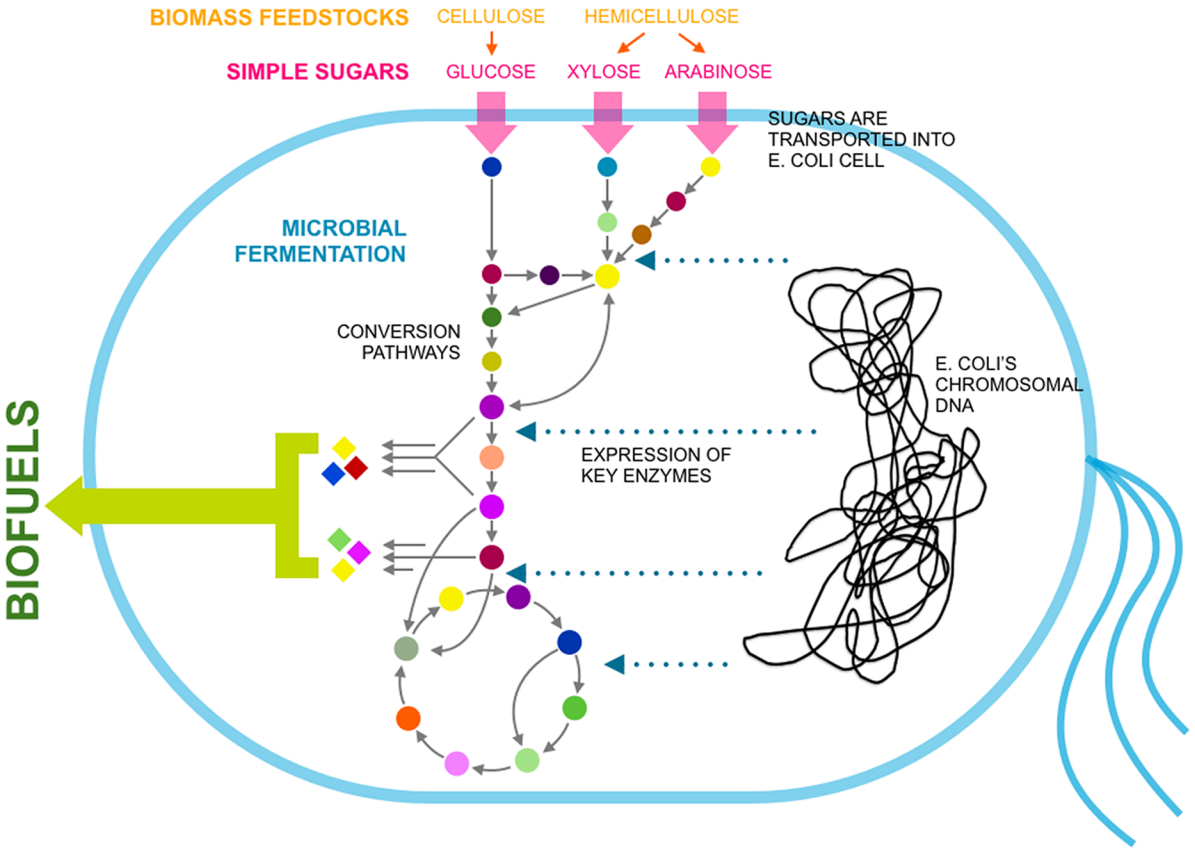


Figure 1. Microbial cell factory for biofuel production. Production of biofuels begins with the conversion of lignocellulosic biomass into simple sugars. Engineered microbes then convert these sugars to advanced biofuels compounds, which can be used as automotive gasoline, diesel, and jet fuels. This Review focuses on the latter portion, specifically on recent advances in the engineering of microbial strains that convert simple sugars to advanced biofuel compounds.

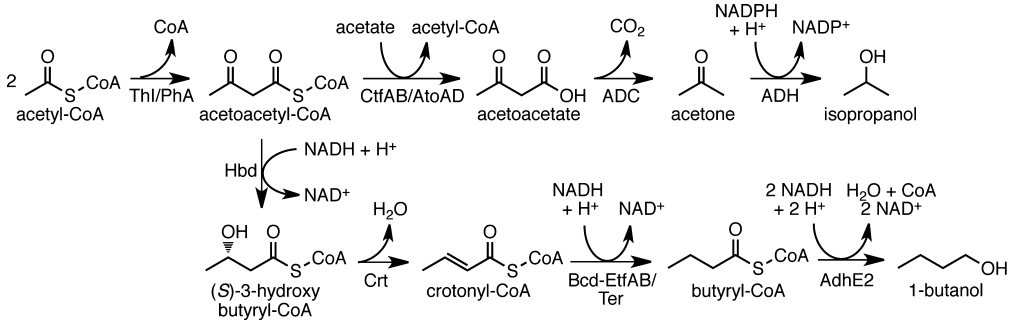


Figure 2. Biosynthetic pathways for fermentative short-chain alcohol production.

Table 1. Summary of Titers and Yields for Select Fuels

fuel	pathway used	host organism	titer (g/L)	yield (%)	ref
isopropanol	alcohol (fermentative)	*E. coli*	143	67	6
1-butanol	alcohol (fermentative)	*E. coli*	30	70	11
isobutanol	alcohol (non-fermentative)	*E. coli*	50	68	14
branched alcohols[a]	alcohol (from protein hydrolysates)	*E. coli*	4.0	56	25
FAEE	fatty acid	*E. coli*	0.674	9.4	36
FAEE	fatty acid	*E. coli*	1.5	28	40
methyl ketones	fatty acid	*E. coli*	0.38	58	42
alkanes/alkenes	fatty acid	*E. coli*	0.3	3.5	43
farnesol	isoprenoid	*E. coli*/*S. cerevisiae*	0.135/0.145	ND	57/55
bisabolene	isoprenoid	*E. coli*/*S. cerevisiae*	0.9	4	67

[a]Includes isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol.

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498–513

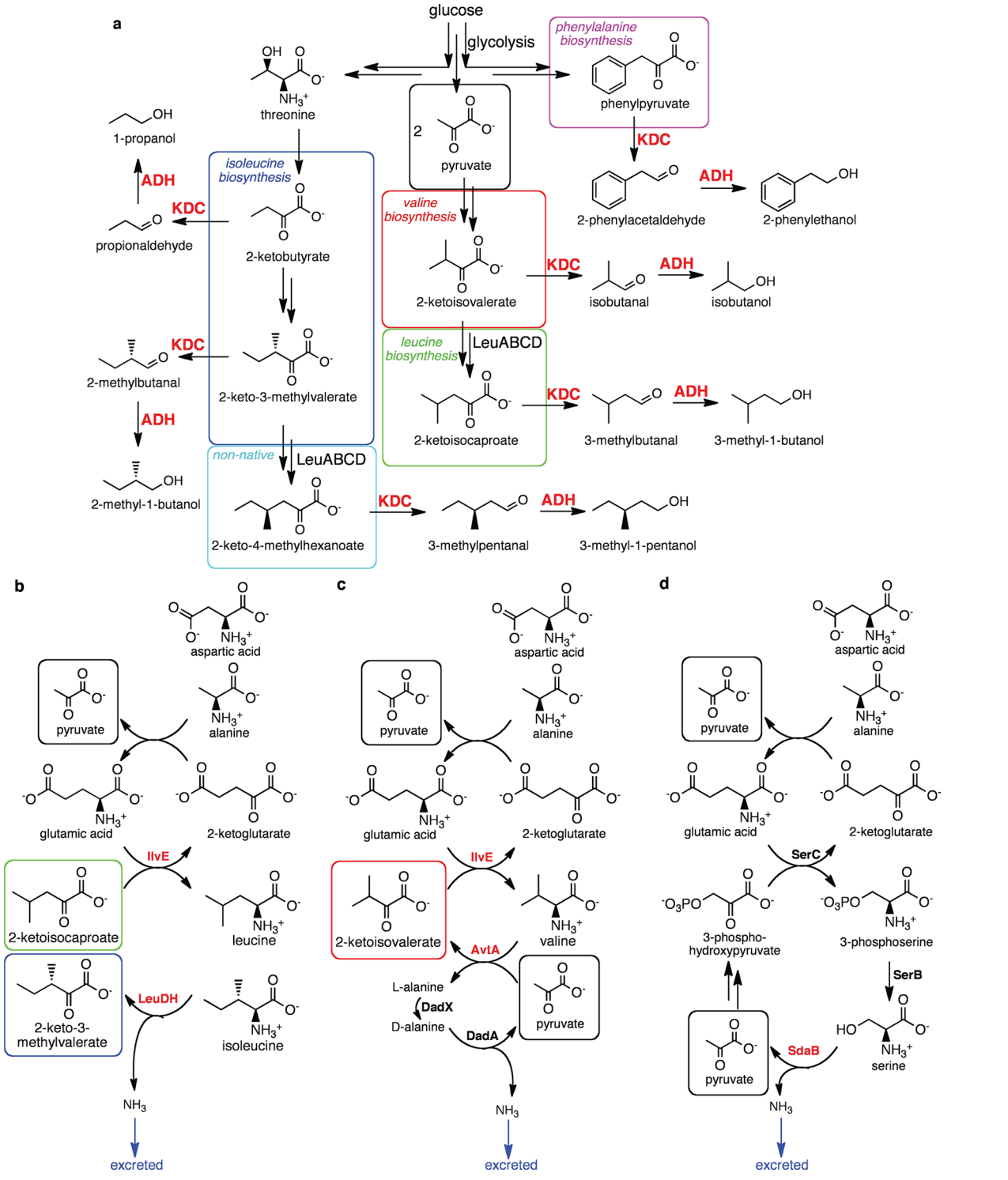


Figure 3. Non-fermentative 2-ketoacid-derived biosynthetic pathways for alcohol production. (a) The overall 2-ketoacid scheme, based on amino acid biosynthesis (2-ketoacids in colored boxes): KDC, ketoacid decarboxylase; ADH, alcohol dehydrogenase. (b-d) Rerouting the nitrogen flux in bacteria to produce C_4 and C_5 alcohols from proteins. Transamination and deamination cycles engineered to convert amino acids, those that cannot be directly deaminated, to 2-ketoacids, pyruvate, and ammonia. In all three cycles, the amino groups in aspartic acid and alanine are transferred to 2-ketoglutarate to yield pyruvate and glutamic acid. (b) IlvE transfers the amino group from glutamic acid to either 2-ketoisocaproate or 2-keto-3-methylvalerate to yield leucine or isoleucine, respectively. Finally, LeuDH from *Thermoactinomyces intermedius* deaminates leucine and isoleucine to yield 2-ketoisocaproate or 2-keto-3-methylvalerate, respectively. (c) IlvE transfers the amino group from glutamic acid to 2-ketoisovalerate to yield valine. AvtA subsequently transfers the amino group from valine to pyruvate to yield 2-ketoisovalerate and L-alanine. Finally, DadX isomerizes L-alanine to D-alanine, which is deaminated to yield pyruvate. (d) SerC transfers the amino group from glutamic acid to 3-phosphohydroxypyruvate to yield 3-phosphoserine. SerB converts 3-phosphoserine to serine, which is subsequently deaminated to yield pyruvate. In all three transamination and deamination cycles, NH_3 is excreted out of the cells and cannot be reassimilated because gdhA and glnA, the two genes responsible for nitrogen assimilation, have been deleted. Excretion of NH_3 drives the cycles toward 2-ketoacid/pyruvate production.

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498–513

fermentative and non-fermentative pathways in host microorganisms.

Fermentative Short-Chain Alcohol Fuels. The short-chain alcohols isopropanol and 1-butanol have higher energy density and lower hygroscopicity than ethanol and have been produced in engineered hosts. The synthesis of isopropanol has been achieved in *Escherichia coli*[4,5] using a fermentative pathway (Figure 2) that begins with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. The CoA group is subsequently transferred to acetate, resulting in the formation of acetoacetate and acetyl-CoA. Decarboxylation of acetoacetate yields acetone, which is then reduced to form isopropanol. The enzymes in this biosynthetic scheme are from *Clostridium acetobutylicum* (Thl, CtfAB, and ADC), *Clostridium beijerinckii* (ADH), and *E. coli* (AtoAD). Expression of these enzymes in *E. coli* resulted in production of isopropanol at titers from 4.9 to 13.6 g/L depending on the glucose concentration in the media. At these concentrations, isopropanol is toxic to *E. coli*, so to alleviate toxicity and to further improve the production titer, isopropanol was continuously removed from production media via gas trapping. Notably, this strategy led to an isopropanol titer of 143 g/L after 240 h (Table 1), or 67% of the theoretical yield,[6] significantly higher than the production titer of the native clostridial strain, which was only 2 g/L.

Due to its high energy density and low hygroscopicity, 1-butanol has also emerged as an attractive biofuel target for microbial metabolic engineering, particularly engineering in *E. coli* and *Saccharomyces cerevisiae*. The native 1-butantol biosynthetic pathway in *C. acetobutylicum* and other related clostridial species begins with the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA (Figure 2). In a similar fashion to fatty acid biosynthesis, acetoacetyl-CoA is converted to butyryl-CoA via reduction, dehydration, and a second reduction. Finally, butyryl-CoA undergoes two rounds of NADH-dependent dehydrogenation to form 1-butanol. Expression of this clostridial pathway in a two-plasmid *E. coli* system led to low levels of 1-butanol production, 13.9 mg/L in 40 h.[7] Subsequent gene deletions that reduce lactate, ethanol, acetate, and succinate side-products gave an increase in titer to 373 mg/L, and when this strain was grown in Terrific Broth-enriched, glycerol-supplemented medium, 1-butanol production increased further to 552 mg/L. Eliminating competing pathways for carbon and reducing cofactor usage also improved production titer to 1.2 g/L in another study.[8] In an investigation of 1-butanol production in multiple microbial hosts, relatively low yields in the range of 34 mg/L were encountered following polycistronic expression of the clostridial pathway genes in *E. coli*.[9] Individual expression of these genes enhanced production to 200 mg/L. The production titer was further improved to 580 mg/L upon elevation of the glycolytic flux. Specifically, *S. cerevisiae* formate dehydrogenase and *E. coli* native glyceraldehyde 3-phosphate dehydrogenase were overexpressed along with the 1-butanol biosynthetic pathway.

More recently, Bond-Watts et al. described another improvement in the 1-butanol production titer by carefully considering the mechanism of the individual enzymes in the pathway and replacing enzymes that are naturally reversible with those that drive the reaction toward 1-butanol formation.[10] First, the *thl* gene, which encodes for acetoacetyl-CoA thiolase/synthase, was replaced with *phaA*, the isozyme from *Ralstonia eutropha*. PhaA is believed to be highly efficient at condensing acetyl-CoA to acetoacetyl-CoA as demonstrated by its capability to support polyhydroxyalkanoate (PHA) production at high levels in the native host *R. eutropha*. Second—and key to their success—the introduction of an irreversible step drives the pathway toward 1-butanol production. Specifically, the *bcd-etfAB* genes, which encode for butyryl-CoA dehydrogenase and two redox partners, were replaced by the *ter* gene from *Treponema denticola*, which encodes for crotonyl-CoA reductase. This replacement effectively creates a kinetic trap that catalyzes the irreversible conversion of crotonyl-CoA into butyryl-CoA. Notably, the 1-butanol yield in the engineered strain employing this strategy reached 2.95 g/L, which was further improved to 4.65 g/L (28% of the theoretical yield) when the *E. coli* pyruvate dehydrogenase complex was overexpressed to increase the availability of the starting material acetyl-CoA and the reducing equivalent NADH. These studies highlight the importance of enzymological inquiry for driving more optimal design of the pathway.

Similarly, an anaerobic, NADH-dependent pathway was used to force cells to drive 1-butanol production forward in a separate study.[11] The NADH-utilizing *ter* substitution mentioned above was used in conjunction with overexpression of formate dehydrogenase, which relieves excess pyruvate and forms CO_2 and NADH, providing additional reducing equivalents. A phosphate acetyltransferase was deleted to discourage acetate production and cause a buildup of acetyl-CoA, and an acetyl-CoA acetyltransferase with higher activity was chosen to more efficiently catalyze acetoacetyl-CoA formation. This strategy allowed an increase in 1-butanol titer to 15 g/L over 3 days in flasks (88% of the maximum theoretical yield), and 30 g/L with continuous gas stripping over 7 days in a fermentor (70% of the theoretical maximum).

S. cerevisiae has also been explored as a production host for 1-butanol production, though with relatively moderate results. Using a combinatorial approach, whereby isozymes of the 1-butanol biosynthetic pathway from different organisms were substituted for the clostridial enzymes, Steen et al. were able to engineer a strain that produces 1-butanol at a titer of 2.5 mg/L.[12] Though this level of production is significantly lower than the levels produced in engineered *E. coli*, the authors identified key bottlenecks in the pathway using metabolite analysis and provided a clear path toward improving the yield.

Non-fermentative Alcohol Fuels. A second pathway was devised by Liao and co-workers to produce longer (up to C_5) and branched-chain alcohols by hijacking the native amino acid metabolism of the host (Figure 3a). Amino acid biosynthesis involves 2-ketoacid intermediates, which may be converted to aldehydes by the expression of a heterologous ketoacid decarboxylase (KDC). An alcohol dehydrogenase (ADH) then reduces the aldehyde to the alcohol product. In the initial deployment of this approach, the alcohols isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol were produced in *E. coli*.[13] Further metabolic engineering enhanced the yield; overexpression of genes responsible for the biosynthesis of the L-valine precursor 2-ketoisovalerate (C_5), in conjunction with substitutions of genes from other organisms and deletion of several genes from competing pathways, resulted in the production of up to 22 g/L isobutanol in shake flasks, 86% of the theoretical maximum. Using the same strain grown in a bioreactor outfitted with a gas-stripping system, 50 g/L isobutanol was produced after 72 h, corresponding to 68% of the theoretical maximum.[14] Ensuing studies used additional metabolic engineering to tailor the production of individual alcohols and increase their yields. Here, strategies including gene deletions, enzyme substitutions,

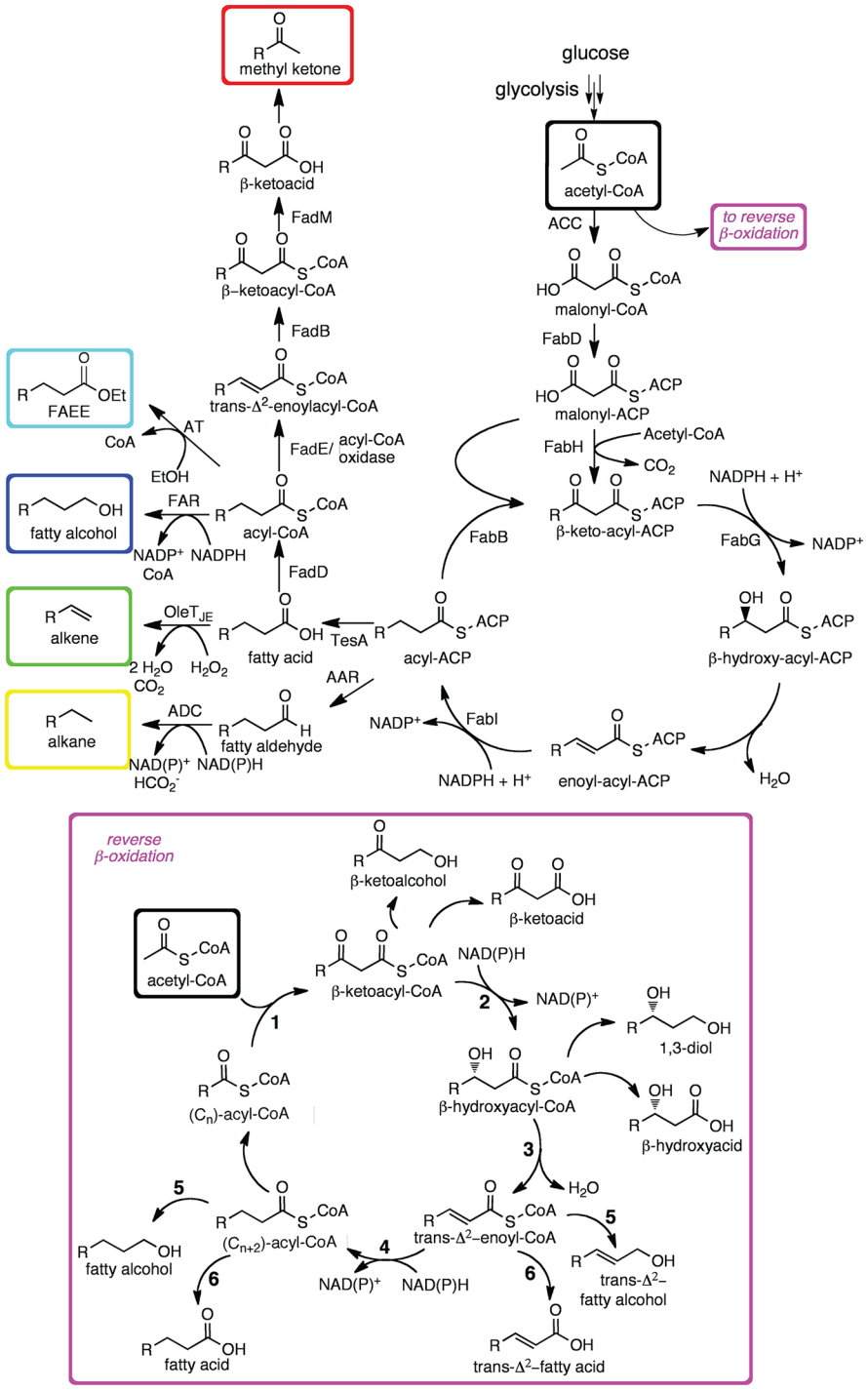


Figure 4. Production of fatty acid-derived biofuels. (a) The native fatty acid pathway in *E. coli* is engineered to produce biofuels (colored boxes). ACC, acetyl-CoA carboxylase; FabD, malonyl-CoA:ACP transacylase; FabH, *β*-keto-acyl-ACP synthase III; FabG, *β*-keto-acyl-ACP reductase; FabZ, *β*-hydroxyacyl-ACP dehydratase; FabI, enoyl-acyl-ACP reductase; FabB, *β*-keto-acyl-ACP synthase I; TesA, acyl-ACP thioesterase; FadD, acyl-CoA synthase; FadE, acyl-CoA dehydrogenase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadM, thioesterase; FadA, 3-keto-acyl-CoA thiolase; FAR, acyl-CoA reductase; AT, a nonspecific acyltransferase/wax-ester synthase; AAR, acyl-ACP reductase; ADC, aldehyde decarbonylase; $OleT_{JE}$, a cytochrome P450 enzyme that reduces fatty acids to terminal alkenes. (b) Reversal of the *β*-oxidation cycle to produce biofuels. **1**, thiolase (yqeF, fadA); **2**, *β*-hydroxyacyl-CoA dehydrogenase (fadB); **3**, enoyl-CoA hydratase (fadB); **4**, enoyl-CoA reductase (ydiO). Intermediates in the pathway are converted to a diverse set of molecules using **5** (acyl-CoA reductases and alcohol dehydrogenases) and **6** (acyl-CoA thioesterases).

and random mutagenesis enhanced the yield or host tolerance of alcohols including 1-propanol and 1-butanol,[15,16] isobutanol,[17,18] 2-methyl-1-butanol,[19] and 3-methyl-1-butanol.[20]

Even longer-chain alcohols (>C_5) were made accessible following the introduction of an elongation step catalyzed by LeuABCD (Figure 3a).[21] The native substrate for LeuABCD is 2-ketoisovalerate (C_5), which is first lengthened by two carbon atoms from acetyl-CoA and then decarboxylated to 2-ketoisocaproate (C_6). However, LeuABCD was found to be promiscuous enough to tolerate 2-keto-3-methylvalerate (C_6),

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498–513

which is one methyl group longer than the native substrate, resulting in the production of a 2-keto-4-methylhexanoate (C_7) intermediate that gave C_6 alcohols following the action of KDC and ADH. Then, structure-guided rational design of LeuA, KDC, and ADH was carried out based on the previously solved crystal structures of homologous enzymes, with the goal of enlarging the substrate binding pockets to enhance the yield of C_6 alcohols. Not only was the production of C_6 alcohols increased by several-fold, elongation by up to two additional carbon atoms was also attained, resulting in the production of alcohols up to C_8.[21]

Biofuel production based on the KDH- and ADH-mediated 2-ketoacid pathway has also been transferred from *E. coli* to other hosts. *Corynebacterium glutamicum* is used in the industrial production of amino acids and has been engineered to produce up to 4.9 g/L of isobutanol, among several C_3−C_5 alcohols.[22] Bypassing the need for deconstruction to simple sugars, *Clostridium cellulolyticum* can grow directly on crystallized cellulose and was engineered using the same strategy to produce isobutanol from cellulose in titers of up to 0.66 g/L.[23] To further bypass even cellulose biosynthesis, the cyanobacterium *Synechococcus elongatus* was engineered to channel carbon fixed from CO_2 to pyruvate, through L-valine metabolism, and finally to isobutanol, following expression of heterologous KDC and ADH.[24] Due to the relative intolerance to isobutanol compared to isobutyraldehyde, the ADH substrate, production of isobutanol reached only 0.45 g/L, while the maximum titer of isobutyraldehyde was 1.1 g/L in a strain lacking ADH.

The conversion of protein hydrolysates into C_4 and C_5 alcohols is another inventive strategy to produce short-chain alcohols (Figure 3b−d).[25] Proteins, in comparison to raw materials like lignocellulose and lipids, lack the recalcitrance and dewatering problems that hamper the conversion of lignocellulose and algal lipids into biofuels. Moreover, proteins are highly abundant in rapidly growing *E. coli*. Protein hydrolysates contain a mixture of peptides and amino acids that are readily obtained by proteolytic treatment of cell biomass and can be used directly by microorganisms. In order to efficiently convert protein hydrolysates to biofuels, Huo et al. first deleted two ammonium assimilation genes, *gdhA* and *glnA*, to eliminate ammonia reuptake and drive the flux of nitrogen toward deamination. The authors then introduced three transamination and deamination cycles to convert amino acids to 2-ketoacids, pyruvate, and ammonia, whose excretion directs nitrogen flux out of the cells, driving the deamination reactions to completion (Figure 3b−d). The three 2-ketoacids produced, namely, 2-keto-methylvalerate (KMV), 2-ketoisocaproate (KIC), and 2-ketoisovalerate (KIV), are subsequently converted to ethanol (EtOH), isobutanol (iBOH), 2-methyl-1-butanol (2MB), and 3-methyl-1-butanol (3MB) via the engineered 2-ketoacid pathway described above. Using this strategy, *E. coli* produced a mixture of iBOH, 2MB, and 3MB in titers up to 4.0 g/L (56% of the theoretical yield) from a modified M9 medium containing approximately 22 g/L of amino acids from yeast extracts. Notably, the authors also demonstrated using algal and bacterial proteins as a feedstock for biofuel production. The proteins were obtained through relatively straightforward proteolytic hydrolysis and heat treatment of algal and bacterial biomass. Even without optimization of the hydrolysate composition, biofuel yields were approximately half of what was achieved using yeast extracts as the protein source. It will be interesting to see how this strategy of using proteins as a raw material for biofuel production could be applied at an industrial scale, specifically, how protein biomass can be isolated from algal and bacterial sources in large quantities. Nevertheless, this strategy provides an exciting new solution to address the problem of nitrogen recycling.

Fatty Acid-Derived Fuels. Fatty acid derivatives are promising biofuel candidates, owing to their high energy density and low water solubility. Therefore, fatty acid biosynthesis has emerged as an important engineering target for the production of transportation fuels from renewable sources. In the phospholipid form, fatty acids are a major component of cell membranes in all organisms. Some organisms, particularly certain species of yeasts and microalgae, can accumulate fatty acids in the neutral form as triacylglycerols (TAG), at up to 30−70% of dry cell weight.[26] While free fatty acids and TAGs are valuable, they cannot be used directly as fuels and must first be converted to fatty acid alkyl esters, fatty acid-derived alkanes, alkenes, alcohols, or methyl ketones. Thus, a common strategy in the microbial production of fatty acid-derived biofuels is to convert fatty acids to the desired fuels *in vivo* and bypass TAG production.

Largely because fatty acids are an integral part of all living organisms, their biosynthesis and regulation have been comprehensively studied in both prokaryotes and eukaryotes.[27−30] In *E. coli*, fatty acid biosynthesis consists of 10 distinct enzymes, which are encoded by a series of separate genes, in the dissociated type II fatty acid synthase (FAS) system.[28] These enzymes, FabA, FabB, FabD, FabF, FabG, FabH, FabI, FabZ, ACP, and TesA, convert 1 equiv of acetyl-CoA and 6−8 equiv of malonyl-CoA into C_{14}−C_{18} fatty acids (Figure 4). Each round of chain elongation requires two reducing equivalents. Malonyl-CoA is produced by acetyl-CoA carboxylase (ACC), which comprises four proteins, AccA−D. In *S. cerevisiae*, *de novo* fatty acid biosynthesis also requires acetyl-CoA carboxylase (ACC) and the FAS complex.[30] However, unlike *E. coli*, yeast FAS complexes are integrated multienzymes that contain the various catalytic domains on two separate multifunctional proteins of comparable size (integrated type I FAS multienzymes). The yeast FAS complex is a 2.6 MDa protein consisting of two nonidentical, multifunctional subunits, α and β, which organize into a $\alpha_6\beta_6$ hexamer.[29] The α subunit, encoded by the *FAS2* gene, contains β-ketoacyl synthase (KS), β-ketoacyl reductase (KR), and acyl carrier protein (ACP) domains. The β subunit, encoded by the *FAS1* gene, contains acetyl-, malonyl-, and palmitoyl-transferase (AT and MPT), as well as dehydratase (DH) and enoyl reductase (ER) domains.

Decades of studies on their biosynthesis and regulation have allowed fatty acids to become attractive metabolic engineering targets. Numerous laboratories have engineered *E. coli* to overproduce free fatty acids in high yields. Common strategies in the majority of engineering attempts thus far include (1) the overexpression of ACC to increase the flux of malonyl-CoA, (2) the overexpression of either the endogenous or heterologous acyl−acyl carrier protein (ACP) thioesterase to relieve feedback inhibition, and (3) the elimination of the β-oxidation pathway that breaks down fatty acids to acetyl-CoA. Combining these strategies has led to a production titer of up to several grams of fatty acids per liter per day.[31−33]

Overproduction of Biodiesels. Over 1 billion gallons of biodiesel, a renewable alternative to diesel fuel, is produced per year in the US alone.[34] Composed of fatty acid methyl and ethyl esters (FAMEs and FAEEs, respectively), biodiesel is

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498−513

traditionally derived from the chemical transesterification of plant oils and animal fats.[35] Recently, Steen et al. engineered *E. coli* to produce $C_{12}-C_{18}$ FAEEs directly from glucose at a titer of 674 mg/L, which is 9.4% of the theoretical yield.[36] To achieve this titer, five engineering strategies were incorporated into the FAEE producing strain: (1) the overexpression of thioesterases to form free fatty acids, (2) the overexpression of acyl-CoA ligases to form fatty acyl-CoA, (3) the elimination of the β-oxidation pathway to prevent fatty acyl-CoAs breakdown, (4) the introduction of an ethanol pathway, and (5) the expression of a nonspecific acyltransferase. Replacement of the acyltransferase with a fatty acyl-CoA reductase yielded medium chain fatty alcohols in titers of up to 60 mg/L. Alternatively, expression of both acyltransferase and fatty acyl-CoA reductase yielded a variety of wax esters. In a move toward consolidated bioprocessing (engineering a microorganism that is capable of both digesting biomass and converting the resulting sugars into biofuels), the group engineered the biofuel-producing strain to express and secrete xylanases that break down xylan into xylose, a pentose that is readily catabolized by *E. coli*.[36] More recently, a biodiesel-producing strain was engineered to express cellulase, xylanase, β-glucosidase, and xylobiosidase enzymes, allowing it to produce FAEEs directly from cellulose and/or hemicellulose components of ionic liquid-pretreated biomass.[37]

S. cerevisiae has also been exploited as a production host for biodiesel production. However, unlike *E. coli*, which does not naturally produce TAGs, *S. cerevisiae* accumulates TAGs in levels of up to 5% of their dry cell weight to function as storage lipids.[38] Therefore, in order to produce FAEEs in yeast, the flux of fatty acids must be diverted from the TAG pathway and into the heterologous FAEE pathway. To this end, Kalscheuer et al. expressed a nonspecific acyltransferase in a *S. cerevisiae* mutant strain that is incapable of producing TAGs.[39] The resulting strain not only reacquires TAG production capability but also can produce FAEEs and fatty acid isoamyl esters (FAIEs).

While it is essential to establish a heterologous pathway (e.g., FAEE pathway) in the production host, further improving production titers to industrially relevant levels remains a challenge. A new development that addresses this problem is the incorporation of regulatory components of gene expression to optimize production titers of heterologous pathways. Notably, Zhang et al. designed a dynamic sensor-regulator system (DSRS) that utilizes *E. coli*'s native sensor for fatty acids and fatty acyl-CoAs (FadR) to dynamically regulate the expression levels of key genes in the engineered biodiesel production pathway.[40] When fatty acid is absent, FadR binds to its DNA binding site in a promoter and hinders the action of RNA polymerase. This causes a repression in transcription of biodiesel pathway genes. However, in the presence of fatty acid/fatty acyl-CoA, FadR instead binds to fatty acyl-CoA and opens up the promoter, allowing RNA polymerase binding, turning on transcription of biodiesel pathway genes. The ability to dynamically regulate the expression levels of these genes in response to a key metabolic intermediate allows for optimized expression of enzymes at levels that do not overburden the cells. The system also helps the cells maintain metabolic balance while preventing the buildup of the toxic fatty acyl-CoA intermediate. The integration of DSRS into biodiesel production led to a 3-fold improvement in the biodiesel titer (1.5 g/L, 28% of the theoretical yield) and increased the stability of the production hosts. Such a strategy can be applied to the microbial production of numerous biofuels and chemicals where natural sensors for a key intermediate exist.

Exploiting the β-Oxidation Pathway. Although the native pathway may be the most obvious strategy to start with, exploiting enzymes beyond their normal functions can also yield excellent results. Gonzalez et al. exploited the reversibility of degradative thiolases, which naturally break down fatty acyl-CoA to acetyl-CoAs in the β-oxidation pathway, and operated the enzymes in the synthetic direction (i.e., carbon-chain elongation) in *E. coli* (Figure 4).[41] In the absence of the natural fatty acid substrates and in the presence of acetyl-CoA from glycolysis, enzymes in the β-oxidation pathway are forced to elongate acetyl-CoA by two carbon units. When combined with endogenous *E. coli* dehydrogenases and thioesterases, the engineered strain produces 1-alcohols, free fatty acids, 3-hydroxy-, 3-keto-, and trans-Δ^2-carboxylic acids in yields that are similar or superior to those of previous strategies. The engineered pathway also does not require ATP-dependent activation of acetyl-CoA to malonyl-CoA and confers superior carbon and energy efficiency.

Another use of the β-oxidation pathway in biofuel production is the production of methyl ketone biofuels in *E. coli*.[42] To produce methyl ketones in *E. coli*, Goh et al. first increased the production of β-ketoacyl-CoA by overexpressing both a heterologous acyl-CoA oxidase and the endogenous FadB and by deleting *fadA*. Overexpression of the endogenous thioesterase (FadM) converts β-ketoacyl-CoA to the β-ketoacid, which subsequently undergoes decarboxylation to form the methyl ketone. Combining these genetic modifications with the use of a decane overlay improved the $C_{11}-C_{15}$ methyl ketone titer to 380 mg/L, or 58% of the theoretical maximum.

Production of Alkanes and Alkenes. Alkanes and alkenes are the predominant components of gasoline, diesel, and jet fuels. The microbial production of alkanes and alkenes has recently been made possible by the discovery and biochemical characterization of an alkane biosynthesis pathway in cyanobacteria.[43] The proposed pathway begins with the NAD(P)H-dependent reduction of fatty acyl-ACPs to free fatty aldehydes. Removal of the terminal carbonyl group yields odd-chain alkanes and alkenes. This second reaction, the aldehyde decarbonylation, is catalyzed by a novel non-heme di-iron enzyme. Detailed studies regarding the mechanism employed by this enzyme are ongoing, including metal-, redox-, and oxygen-dependency.[44−46] Nevertheless, *E. coli* engineered to heterologously express the alkane operon produces a variety of $C_{13}-C_{17}$ alkanes and alkenes, with pentadecane and heptadecene predominating at titers over 300 mg/L when the bacteria are cultured in a modified mineral medium.

A distinct pathway for alkene production involving the head-to-head condensation of fatty acids to form long-chain alkenes ($C_{23}-C_{33}$) has been characterized in *Micrococcus luteus* and *Shewanella oneidensis*. The key enzyme in the pathway, OleA, is proposed to catalyze a thiolase-type reaction. Heterologous expression of these alkene operons in *E. coli* confers on the strain the ability to produce long-chain alkenes, predominantly 27:3 and 29:3 chains.[47] A biosynthetic pathway for a terminal alkene (α-olefin) has also been recently elucidated.[48] The key enzyme in the pathway, OleT, is a cytochrome P450 enzyme from the cyp152 family, which also includes fatty acid hydroxylases. While the exact mechanism for the decarboxylation resulting in alkene formation has not yet been elucidated, heterologous expression of this enzyme ($OleT_{JE}$) in *E. coli* conferred on the organism the ability to produce

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498−513

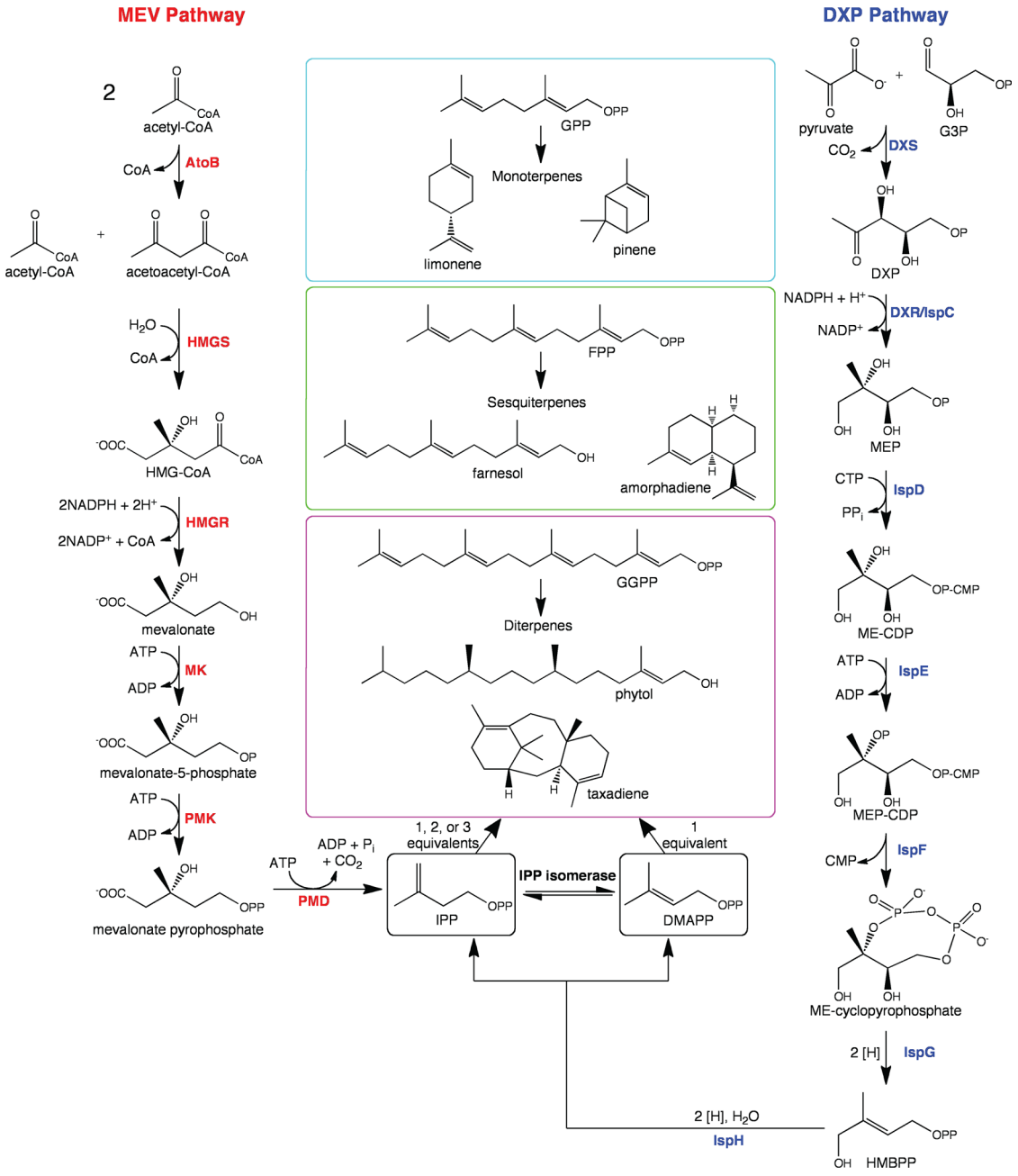


Figure 5. Biosynthetic pathways toward isoprenoids and isoprenoid fuels. Enzymes of the mevalonate (MEV) pathway in red and enzymes of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway in blue. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, phosphomevalonate decarboxylase; G3P, glyceraldehyde-3-phosphate; DXS, DXP synthase; DXR, DXP reductase; MEP, 2-C-methylerythritol 4-phosphate; CTP, cytidine triphosphate; ME, 2-C-methyl-D-erythritol, CDP, cytidine diphosphate; MEP-CDP, ME-2-phosphate-CDP; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranygeranyl pyrophosphate.

terminal alkenes, predominantly 1-pentadecene and 1,10-heptadecadiene.

Isoprenoid-Derived Fuels. Isoprenoids, also called terpenes, are a large and highly varied class of organic compounds that range in biological function to include vitamins, light-harvesting pigments, hormones and sterols, cell defense molecules, electron carriers, and, in archaea, cell membrane phospholipids. Despite their vast structural and functional diversity, all isoprenoids are synthesized by the universal, isomeric, five-carbon (C_5) precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are produced by one of two pathways (Figure 5). The mevalonate (MEV) pathway first involves condensation of three molecules of acetyl-CoA to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) and subsequent reduction to MEV by HMG-CoA reductase, the target of statins such as Lipitor (Pfizer, Inc.), used for reducing blood cholesterol levels. From MEV, three phosphorylations and one decarboxylation produce IPP, and isomerization to DMAPP completes the pathway. The non-mevalonate pathway, also called the 2-C-methyl-D-erythritol-4-phosphate (MEP) or 1-deoxy-D-xylulose-5-phosphate (DXP) pathway, bypasses acetyl-CoA and begins

dx.doi.org/10.1021/sb300074k | *ACS Synth. Biol.* 2012, 1, 498–513

at glyceraldehyde-3-phosphate and pyruvate, which are first condensed to DXP and then reduced and isomerized to MEP, before attachment to cytidine monophosphate (CMP). This intermediate is phosphorylated and then cyclized, liberating CMP and forming an unusual cyclopyrophosphate intermediate. The final ring-opening and reductive steps produce both IPP and DMAPP and are performed by two enzymes that harbor [4Fe-4S] clusters and employ mechanisms that are unconventional and controversial and may involve organometallic intermediates.[49−51] An isomerase may then interconvert IPP and DMAPP, as in the MEV pathway. With some exceptions, the MEV pathway is present in the cytosol of higher eukaryotes and archaea, while the DXP pathway is used in bacteria and in plant plastids.

Once formed, the IPP and DMAPP precursors are condensed by prenyltransferases to lengthen the carbon chain in C_5 increments. One molecule of DMAPP and one or more molecules of IPP may be linked in a linear, head-to-tail fashion, forming geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}), or geranylgeranyl pyrophosphate (GGPP, C_{20}). Dedicated terpene synthases convert these prenylpyrophosphate intermediates to C_{10} monoterpenes (e.g., limonene and pinene), C_{15} sesquiterpenes (e.g., farnesol and amorphadiene), or C_{20} diterpenes (e.g., phytol and taxadiene) (Figure 4). Terpene synthases that generate cyclic or polycyclic products may also be referred to as terpene cyclases. Terpene synthases are responsible for the wide structural diversity of isoprenoids, as their reactivity determines the structural skeleton of the constructed molecule. Alternatively, prenylpyrophosphate intermediates themselves may be condensed; for example, two FPP molecules are joined "head-to-head" to form squalene, the triterpene (C_{30}) precursor to the steroids, which include cholesterol, estradiol, testosterone, and ergosterol. Finally, additional enzymes may further modify or decorate the isoprenoid scaffold, giving a final layer of structural complexity.

Both the intermediates and the final products of isoprenoid pathways are good candidates for advanced biofuel compounds. Shorter, branched isoprenoid molecules can be used in gasoline, while larger, cyclic isoprenoids may be suitable for diesel or jet fuel. The vast range of hydrocarbon architectures accessible from the same pathway is a particular advantage, as incorporation of different terpene synthases into the same isoprenoid-producing host strain can yield completely different products. For isoprenoid prenylpyrophosphate intermediates, hydrolysis of IPP to isopentenol has been performed in *E. coli* using a pyrophosphatase from *Bacillus subtilis*.[52] Farnesol, present in essential oils such as citronella, lemongrass, and rose oils, has been produced in *S. cerevisiae* from FPP by multiple strategies,[53] including the use of a native pyrophosphatase,[54] by enhanced expression of MEV pathway enzymes[55] and by repression of the endogenous squalene synthase.[56] Farnesol was also produced in engineered *E. coli* co-expressing the heterologous MEV pathway and FPP synthase, where endogenous phosphatases are believed to be responsible for the final FPP hydrolysis step.[57]

Many isoprenoid products are also being pursued as biofuel targets. The linear sesquiterpene farnesene and its isomers are found in fruits to give a characteristic apple scent, in potatoes as an insect repellent,[58] and in insects as a distress pheromone.[59] Its physicochemical properties also make it a promising fuel candidate.[60] Farnesene (Biofene, Amyris Biotechnologies, Inc.) has been produced in both engineered *E. coli* and *S. cerevisiae* from FPP using a farnesene synthase and is currently being pursued for mass production as a fuel additive.[60] Farnesene may also be chemically hydrogenated to fully saturated farnesane, which has greater oxidative stability and higher cetane number.[60,61]

Pinene is a bicyclic monoterpene that occurs naturally in pine resin, forming the primary constituent of turpentine. The properties of pinene dimer mixtures are similar to those of jet fuel.[62] Low-level pinene production has been demonstrated in engineered *E. coli* using the endogenous DXP pathway coupled with a nonnative GPP synthase and monoterpene synthases.[63] Pinene, among other isoprenoids, was also produced in engineered *S. cerevisiae* in small quantities using the endogenous MEV pathway with modified promoters in combination with terpene synthases.[64] Limonene, another cyclic monoterpene, is found in citrus fruits to impart a lemony odor and can be used as a jet fuel component.[65] Engineered *E. coli* expressing IPP isomerase, GPP synthase, limonene synthase, and two downstream enzymes produced limonene in small quantities (5 mg/mL).[66] Future metabolic engineering to enhance yields of limonene and pinene may enhance the feasibility of these compounds as biofuels.

Recently, the sesquiterpene bisabolene, found in the essential oils of a variety of plants and herbs, and its fully reduced derivative bisabolane were shown to possess physical and chemical properties that are highly similar to D2 diesel fuel.[67] In this study, bisabolene synthase variants from several plant species were tested for bisabolene production in both *E. coli* and *S. cerevisiae*. Expression in *E. coli* of a codon-optimized bisabolene synthase from the fir tree *Abies grandis*, in conjunction with the introduction of an optimized heterologous MEV pathway, resulted in bisabolene production of >900 mg/L. An FFP overproducing strain of *S. cerevisiae* also gave bisabolene titers of >900 mg/L using the same bisabolene synthase. The crystal structure of this enzyme was recently determined and may be exploited for enzyme engineering to further improve yields in host microbes.[68]

In the future, the pursuit of new isoprenoid-derived biofuel targets is sure to continue, yet further work in metabolic engineering and enzyme characterization is also of significant importance. Much attention is being paid to the optimization of the better-understood MEV pathway. Heterologous expression of the pathway in *E. coli*[69,70] does mitigate otherwise endogenous regulatory effects that may hinder isoprenoid production, but intermediates that are either toxic or downregulate enzymes of pathway are still a concern. Many steps along the MEV pathway are regulated by negative-feedback loops that if overcome could increase yields. For example, accumulation of the heterologous MEV pathway intermediate HMG-CoA in *E. coli* was overcome by modulating HMG-CoA reductase expression, relieving a bottleneck in the pathway.[71] Also, accumulation of isoprenoid prenylpyrophosphate intermediates such as FPP are also known to inhibit cell growth.[69] The more recently elucidated DXP pathway is less understood from biochemical and regulatory standpoints, and heterologous expression of this pathway in *S. cerevisiae* for enhanced isoprenoid production has not yet been shown to be feasible. Yet metabolic engineering of the native DXP pathway in *E. coli* has been used to increase isoprenoid yields. For example, lycopene yields have been enhanced by rerouting DXP pathway precursors,[72] manipulating promoter sequences of DXP pathway enzymes,[73,74] introducing gene deletions,[75,76] and incorporating a dynamic metabolic regulatory system.[77] In

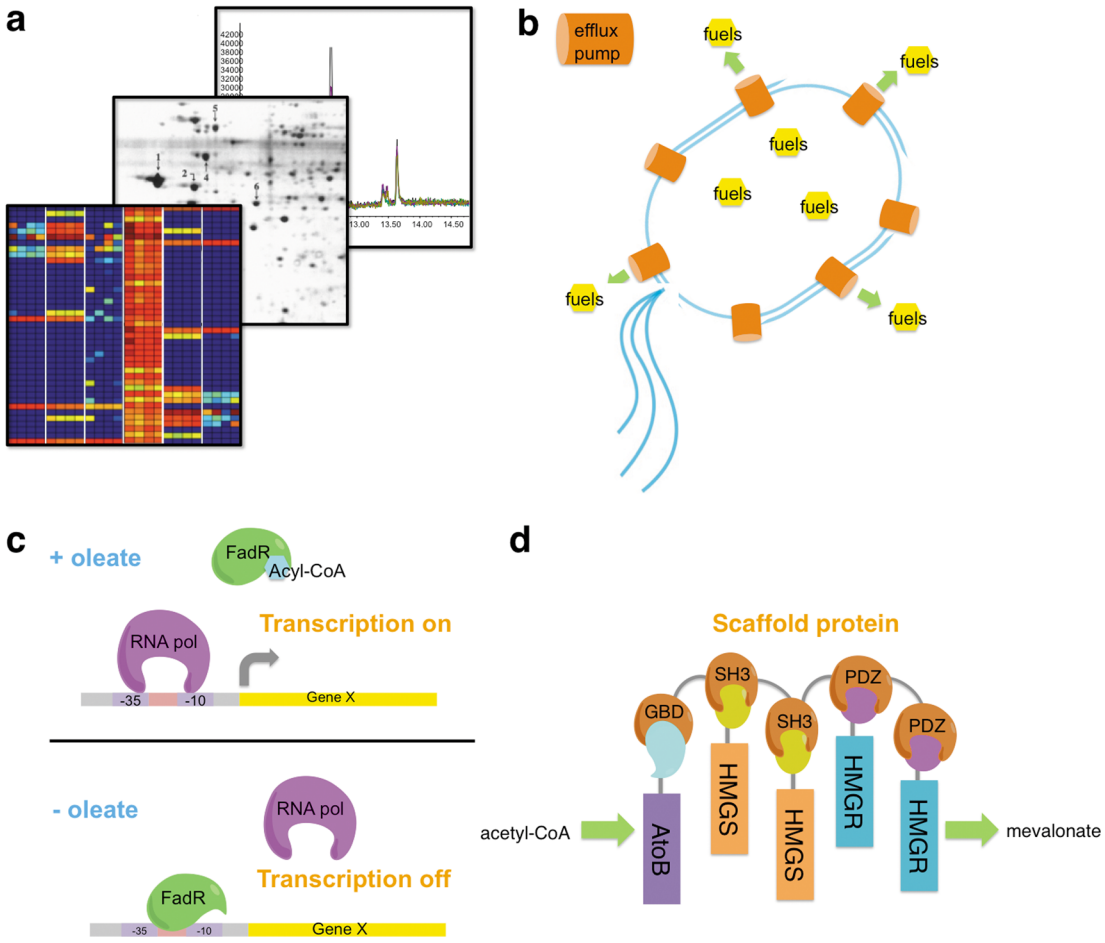


Figure 6. Strategies in strain development for biofuel production. (a) Application of various "omics" analyses (such as metabolomics, proteomics, transcriptomics, and fluxomics) to identify bottlenecks and optimize biofuel titer in the engineered production strain. (b) Overexpression of efflux pumps to alleviate biofuel toxicity. (c) Integration of a dynamic sensor-regulator system into a biofuel production pathway results in more optimal levels of gene expression and higher biofuel titer. (d) Synthetic protein scaffolds facilitate colocalization of key enzymes in a biofuel production pathway and enable fine-tuning of the enzyme stoichiometry to maximize metabolic flux through the pathway.

either the MEV or the DXP pathway, protein expression levels, enzyme activity and stability, and pathway regulation are prime areas for optimization.

STRATEGIES IN STRAIN DEVELOPMENT FOR BIOFUEL PRODUCTION

The nascent fields of synthetic biology and metabolic engineering are inherently multidisciplinary, and it follows that the strategies employed to optimize pathway performance and increase biofuel titers are diverse and manifold. Overall goals include identifying and overcoming metabolic bottlenecks, toxicity, and limits in enzyme activity, and may involve a multitude of techniques. Here, we offer an overview of both commonly used and newly devised strategies that have been employed.

"Omics" Analysis and Metabolic Models. Before metabolic engineering is pursued to maximize strain performance, the identification of metabolic bottlenecks would be ideal. Many factors contribute to pathway productivity, and although random strain engineering can lead to higher product titers, it is difficult to predict which steps along a pathway or which physiological processes in the host are best to target. For example, techniques that allow the monitoring of transcript levels, such as quantitative real-time reverse transcriptase PCR (qRT-PCR) and microarray-based transcriptome analysis (Figure 6a), can be highly useful indicators of gene expression and can provide one level of knowledge to identify potential bottlenecks. However, other factors including mRNA structure, codon usage, translation efficiency, protein folding and activity, protein localization, and protein stability are not taken into account, and further analysis may be pursued. The advent of more facile metabolomic[78] and proteomic[79,80] inquiry can help further pinpoint individual steps of the pathway for targeted manipulation in order to balance gene expression and avoid bottlenecks (Figure 6a). Even very small quantities of a protein, on the order of tens of copies per cell, can be detected from total cell lysates using advances in LC–MS/MS-based single reaction monitoring (SRM).[81] In this way, insufficient expression of individual enzymes, perhaps in conjunction with a buildup of upstream intermediates, can be identified for further metabolic engineering of the strain.

In addition, it is common in metabolic engineering to alter many variables concurrently, where several factors such as gene order, promoter strength, enzyme variant, or plasmid copy number are varied and the product yield is measured. But when an increase in yield fails to materialize in a host strain, it is tempting to simply abandon the strain and perhaps the underlying engineering rationale, discarding potentially useful effects that are masked behind a negative result. However, more

dx.doi.org/10.1021/sb300074k | ACS Synth. Biol. 2012, 1, 498–513

detailed metabolomic, proteomic, and fluxomic study may reveal that a bottleneck has in fact been overcome, only to be met with another challenge. Therefore, "omics" analysis can expose a wealth of valuable information regarding the physiology of a constructed host strain.

In a recent example, protein expression of the isoprenoid MEV pathway in *E. coli* as a function of genetic factors such as promoter strength and codon usage was interrogated using targeted proteomics.[82,83] Among the findings uncovered in these studies were surprisingly low expression levels of mevalonate kinase (MK) and phosphomevalonate kinase (PMK), consecutive enzymes of the MEV pathway (Figure 5), compared to other pathway enzymes. These observations led to the hypothesis that a bottleneck exists in the pathway at mevalonate and phosphomevalonate, and subsequent qRT-PCR analysis for transcript levels indicated the possibility that the mRNA sequences were suboptimal. Subsequent codon optimization of MK and PMK genes for expression in *E. coli* improved both protein expression and isoprenoid production, partially relieving the bottleneck. Previously, all MEV pathway genes were under control of a single promoter, and the insertion of a strong promoter directly upstream of the MK gene gave a further enhancement in isoprenoid yield. In this manner, results from targeted proteomics were able to direct metabolic engineering efforts toward potential bottlenecks.

In addition to "omics" analysis, computational modeling can help tailor a rational approach to metabolic engineering and pathway design. Metabolic models exist that can identify all possible pathways that can lead to the production of a compound of interest and prioritize them according to desired criteria, such as avoiding certain intermediates or limiting the number of individual steps.[84−86] Subsequent metabolic flux analysis (MFA) or flux balance analysis (FBA) can be used to model metabolic behavior *in silico* based on the chosen host genotype, to predict the effect of targeted gene deletions or insertions on the metabolic outcome.[89−93] For instance, MFA-guided metabolic engineering suggested gene insertions that improved the bioethanol yield in *S. cerevisiae*.[87] Likewise, a single gene deletion derived from a genome-wide metabolic model[88] resulted in an 85% increase in yield of the sesquiterpene cubebol in *S. cerevisiae*.[89] More recently, guided by metabolic modeling *E. coli* was engineered to produce 18 g/L of 1,4-butanediol (BDO), the first biosynthetic route to this commodity precursor from a renewable feedstock previously accessible from only petroleum-based precursors.[90] In this example, a genome-wide model[91] and a pathway prediction algorithm[92] considered not only direct metabolic routes toward BDO, but also additional factors such as the balancing of energy and redox requirements and the circumvention of potentially toxic intermediates and side products. In these ways, metabolic modeling can provide the key initial insights into developing new engineering strategies, and its use for microbial production of biofuels holds great promise.

Overcoming Toxicity. Alleviating biofuel toxicity to the host cells is necessary for maximizing the product titer. Toxic molecules trigger stress responses in host cells that may lead to the inactivation of foreign pathway genes, slow cell growth, or even cell death, all of which decrease biofuel yields. Even when the target molecule is not toxic to the producing host, excretion of the compound into the extracellular space can minimize the compound's intracellular concentration, thereby alleviating feedback inhibition.

The overexpression of efflux pumps in the host cell to allow the toxic biofuel to be transported into the media has been explored in recent years as a method to overcome toxicity problems (Figure 6b). Sequenced bacterial genomes include many putative efflux pumps. Using bioinformatics, Dunlop et al. constructed a set of potential solvent-resistant efflux pumps from genomic analysis.[93] To facilitate the screening of these transporter candidates, a competitive growth assay was developed that allowed the authors to distinguish efflux pumps that confer biofuel tolerance to *E. coli* from those that do not. Under the assay conditions, *E. coli* cells expressing functional efflux pumps thrived in the presence of the target biofuel and dominated the population. To demonstrate that the efflux pumps that provide biofuel tolerance also improve biofuel production, a representative beneficial pump was overexpressed in a limonene-producing strain. The engineered strain expressing the efflux pump produced approximately twice the amount of limonene as compared to the control strain with no pump. While it is important to note that the current limonene production level of approximately 60 mg/L is well below toxic levels, these results nevertheless underscore the role of efflux pumps in improving the biofuel production titer.

Modulating Protein Expression. Metabolic engineering of microbes for biofuel production requires selection of the right host as well as identification of all the necessary enzymes in the biosynthetic pathway. Optimally expressing pathway genes to ensure the efficient conversion of starting materials to target molecules is equally critical to the success of any metabolic engineering strategy. Expression at too low a level will lead to the accumulation of potentially toxic pathway intermediates, while expression at too high a level will deplete the host cells of cellular resources (carbon source, energy, and cofactors) that would be diverted to produce supernumerary proteins and nucleotides. Moreover, some heterologous proteins are inherently toxic to the host cells, and overexpression of these proteins may lead to slow growth or even cell death. Given how important optimal expression levels and balanced metabolism are to the overall yield of biofuels, it is not surprising that an extensive amount of work has been done to address this issue. Many approaches have been used, including promoter engineering, altering plasmid copy number, ribosomal binding sites, codon usage, mRNA folding and stability, and the use of RNA devices and dynamic controls of protein expression that actively respond to conditions in the cell. Here, we consider particular types and examples of protein expression modulation.

Promoter Engineering. In one approach, Alper et al. generated a library of promoter mutants exhibiting a broad range of promoter strengths.[74] Specifically, random mutagenesis of the bacteriophage $P_L-\lambda$ promoter yielded approximately 200 promoter mutants with activities that span a 196-fold range as quantified by green fluorescent protein (GFP) fluorescence, where each promoter was used to drive GFP expression in *E. coli*. By applying the characterized library of promoters to engineered lycopene production in *E. coli*, the authors were able to identify the optimal expression levels of key bottleneck enzymes in the pathway that led to maximal lycopene yield. This promoter engineering strategy has also been applied to the eukaryotic host *S. cerevisiae*. Eleven TEF1 promoter mutants exhibiting activities between 8% and 120% that of the wild-type promoter were generated and characterized. Chromosomal replacement of the native glycerol 3-phosphate dehydrogenase (GDP1) promoter of *S. cerevisiae* by

five of these promoter mutants allowed analysis of the impact of GDP1 activity on the glycerol yield. In another notable example, Jensen et al. generated a library of *Lactococcus lactis* promoters with activities spanning a 400-fold range by varying the −35 and −10 regions of the spacer sequences.[94] Schlabach et al. screened every possible sequence for the 10-mer transcription factor binding site upstream of the CMV promoter and identified sequences, or synthetic enhancer elements, that are competent at activating transcription in multiple eukaryotic cell lines.[95]

Ribosomal Binding Site (RBS) Engineering. The ribosomal binding site (RBS) also provides an attractive target for regulation of gene expression. The DNA sequences immediately upstream and downstream of the RBS determine the mRNA secondary structure, which has been shown to affect the rate of translation initiation. Using a thermodynamic model, Salis et al. created a predictive method for rationally engineering RBSs that offer control over protein expression level.[96] Later incorporated into a computer program called the RBS calculator, this method is able to predict translation initiation rates in bacteria and enables control of translation initiation rates spanning over a 100,000-fold range.

RNA Devices. In addition to promoter and RBS engineering, engineering of untranslated regions (i.e., 5′ untranslated and intergenic regions) and genetic circuit designs have been tremendously helpful in microbial engineering to provide static and dynamic controls of gene expression. While these RNA devices hold great potential for metabolic engineering of biofuels in microbes, the paucity of design tools that allow users to incorporate such devices into their engineered pathway with a predictable outcome has hampered its crossover from a proof-of-concept to an industrial-scale utilization. To address this problem, Carothers et al. developed a computer-aided approach for designing RNA devices that can regulate gene expression in a predictive and quantitative manner.[97] The team assembled RNA devices based on ribozymes and metabolite-controlled aptazymes for static and dynamic controls of gene expression, respectively. Key to the success of this method is the incorporation of mechanistic modeling and kinetic RNA folding simulations that take into account the different parameters affecting the output of the device (e.g., gene expression level). Notably, fine-tuning of expression levels could be achieved simply by adjusting a few design variables such as ribozyme folding kinetics or the half-life of the RNA transcript. Using these designed RNA devices, the authors were able to control the flux of *p*-aminophenylalanine, a precursor to bioactive compounds and polymers.

Dynamic Biosensor-Regulators. Although extensive work to regulate gene expression has concerned engineering the RBS and promoter strength, the majority of these strategies only provide static control of gene expression without taking into consideration the conditions that the host cell encounters. Moreover, these strategies require the screening and selection of an optimal strain under a specific condition (for example, a particular growth medium or temperature) that may be suboptimal once the best producing strain is subjected to new conditions. To overcome these limitations, metabolic engineers have looked for inspiration from nature, specifically, the way in which nature has evolved exquisite regulatory mechanisms that can sense and regulate gene expression in response to different conditions. This type of dynamic regulation was first examined by Farmer and Liao in their engineered production of lycopene in *E. coli*.[77] Excess glycolytic flux into acetyl phosphate decreases lycopene production, and so an *E. coli* transcription factor and promoter that can sense acetyl phosphate was used as a sensor for excess glucose flux. Accumulations of intracellular acetyl phosphate led the engineered sensor to activate the transcription of lycopene biosynthetic pathway genes thereby diverting the flux from acetate production to lycopene. The engineered strain containing this dynamic sensor-regulator produced lycopene at a titer that is 18-fold higher than that in the control strain with the lycopene pathway driven by a constitutive promoter. More recently, Zhang et al. applied this dynamic sensor-regulator system to improve production of FAEEs in *E. coli*.[40] Unlike in the earlier work by Farmer and Liao where the sensor molecule is an intermediate from a competing pathway, the biosensor in Zhang's work directly senses the key intermediate in the pathway, namely, the fatty acyl-CoA (Figure 6c).

Modulating Protein Activity. As described above, numerous methods can be employed to alter expression of target genes. However, expression levels do not necessarily correlate with protein activity, the ultimate driver of metabolic flux. Enhanced expression of a poor, unstable, or inactive enzyme, for example, may not result in a sufficient boost in biofuel production. Therefore, engineering of the enzymes themselves can play a key part to optimal pathway performance.

Protein Engineering. The design of enzyme variants with desired characteristics can be pursued, such as those with greater stability, tailored reactivities, and improved kinetics. There are multiple approaches that can be explored to generate these enzyme variants. If atomic resolution structural information of the target protein has been determined by techniques such as X-ray crystallography and NMR spectroscopy, mutations can be designed rationally, taking advantage of the rich and detailed mechanistic insight gained from structural analysis. A strong disadvantage to this approach is, of course, that structures of the enzyme of interest must first be solved. In addition, beneficial mutations may not be immediately apparent upon structural analysis. If structural information is not available, directed evolution can be employed, where libraries of enzyme variants can be generated by random mutagenesis and screened for the desired characteristic.[98−100] A disadvantage of this technique is that a high-throughput screening or selection process must be used, and so this method is not applicable to every enzyme system. It is also possible to use a combined approach, where a known structure indicates specific regions of the protein that may be selectively varied to construct a targeted library of mutants.

In a recent example of protein engineering for biofuel production, the cofactor requirements for 2-ketoacid pathway enzymes were altered.[101] One downside to the use of the 2-ketoacid pathway (Figure 3) is its cofactor requirement, as ADH and ketol-acid reductoisomerase (KARI), involved in 2-ketoisovalerate biosynthesis from pyruvate, both require NADPH which can only be regenerated through the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle. For large-scale industrial production, however, anaerobic conditions are preferred due to the significantly lower operating costs and higher theoretical yields, yet under these settings an overabundance of NADH, and not NADPH, is available through glycolysis. To switch the cofactor requirement for KARI, analysis of the crystal structure of the cofactor-free *E. coli* enzyme aligned with the structure of an NADPH-bound spinach homologue identified five positions for mutation: Arg68, Ala71, Arg76, and Ser78 interacted with the NADPH 2′-

phosphate, while Gln110 appeared to affect the orientation of the adenine moiety. Libraries of site-saturated mutants at each position were then constructed and tested for NADPH and NADH consumption. A library of the combination of the best mutations was then assembled and tested. The best KARI variant was a quadruple mutant (A71S, R76D, S78D, Q110 V), which showed a similar catalytic efficiency (k_{cat}/K_M) as the wild-type, with the Michaelis constant (K_M) for NADH of 30 μM besting the wild-type K_M for NADPH of 40 μM, showing that the cofactor requirement of *E. coli* KARI was successfully switched from NADPH to NADH. Expression of this KARI variant in conjunction with a kinetically improved, NADH-utilizing ADH enabled production of isobutanol at 100% of the theoretical maximum. Such switching of cofactor requirements, though highly challenging in practice, holds vast metabolic and industrial benefits and demonstrates the utility of protein engineering in synthetic biology for biofuel production.

Enhancing Protein Activity through Colocalization. In addition to altering the activity of individual enzymes, protein colocalization can also be employed to optimize combined enzyme activity and metabolic flux. Here, pathway enzymes can be designed to be colocalized in the cell, either by enzyme fusion, protein trafficking, or the use of scaffolding proteins. For example, expression of two-enzyme fusions has enhanced flux through isoprenoid-producing pathways in *S. cerevisiae* and given higher product yields.[102−104] Such a colocalization approach has multiple benefits. First, bringing active sites closer together limits the transit time for pathway intermediates and prevents their accumulation, which could give rise to toxic effects. Restricting free diffusion of intermediates also avoids side-reactions carried out by competing pathways, and unstable intermediates are also protected from prolonged exposure to the bulk solvent. Moreover, intermediates are directed more specifically toward downstream processes, bypassing equilibrium and kinetic restrictions imposed by dependence on bulk concentrations. This benefit is made possible because the effective local concentration of the metabolic intermediate is significantly increased, a strong advantage considering that global concentrations of an intermediate may be lower than the K_M of the downstream enzyme. In addition, because the colocalization of enzymes can be constructed in modular fashion, the stoichiometry of enzymes can be more easily manipulated to achieve a more balanced metabolic flux. Finally, the improved efficiency in flux reduces the need for high protein expression levels, which drain the cell of nucleotides, amino acids, sugars, and energy that may be otherwise used for biomass and biofuel production. In these ways, colocalization allows the activities of pathway enzymes to be synergized for increased yield.

In one development of enzyme colocalization, engineered protein scaffolds (Figure 6d) have been employed to enhance flux through the first three steps of the heterologous MEV pathway (Figure 5) in *E. coli*.[105] Well-studied peptide sequences involved in protein−protein interactions were appended to the termini of AtoB, HMGS, and HMGR, where the following three peptide sequences were used, respectively: a peptide (G) that binds specifically to the GTPase binding domain (GBD) of the actin polymerase switch N-WASP, a peptide (S) that binds the SH3 domain from the mouse protein Crk, and peptide (P) that binds to the PSD95/DlgA/Zo-1 (PDZ) domain from the adaptor protein syntrophin. Corresponding synthetic protein scaffolds were then constructed to bind G, S, and P, where varying numbers of GBD, SH3, and PDZ domains were fused together, joined by nine-residue glycine-serine linkers. When G-, S-, and P-tagged AtoB, HMGS, and HMGR enzymes, respectively, were co-expressed with the engineered scaffold in *E. coli*, mevalonate titers were much improved over the non-scaffolded control. In the best strain, in which the scaffold contained one GBD, two SH3, and two PDZ domains ($G_1S_2P_2$), the mevalonate titer was 77-fold higher than with the scaffold-less strain. Further illustrating the importance of the scaffold, even when pathway enzymes were expressed at saturating inducer concentrations, the non-scaffold pathway still did not attain the mevalonate levels that the $G_1S_2P_2$ strain achieved with only basal expression of pathway enzymes. Studies such as this open the door for new colocalization systems that can enhance protein activities to improve product yields.

CONCLUSION AND FUTURE DIRECTIONS

The past decade has seen an explosion in the production of fuel compounds derived from alcohol, fatty acid, and isoprenoid biosynthesis in engineered microbial hosts. With advances in metabolic modeling and the ongoing discovery of new metabolic networks, the possibilities for new routes toward biofuel biosynthesis now appear virtually limitless. A considerable challenge will be in identifying the individual pathways and fuel targets that hold the greatest promise. Once the pathways are chosen, bottlenecks can be identified with more ease than ever before, and new techniques in the metabolic engineer's toolkit make the balancing of protein expression by static or dynamic controls more tunable and predictable. Meanwhile, protein engineering of pathway enzymes themselves through a combination of rational design, directed evolution, and possibly even *de novo* enzyme design has the power to open the door to more optimized or perhaps new, nonnatural paths to biofuel biosynthesis. As a result, innovative recruitment of metabolic pathways toward novel advanced fuel targets at higher product yields is sure to continue, bringing us ever closer to the widespread adoption of advanced biofuels in the global transportation infrastructure.

AUTHOR INFORMATION

Corresponding Author

*E-mail: jdkeasling@lbl.gov.

Notes

The authors declare the following competing financial interest(s): J.D.K. has financial interests in Amyris and LS9.

ACKNOWLEDGMENTS

We thank Weslee S. Glenn (Department of Chemistry, Massachusetts Institute of Technology and Department of Biological Chemistry, John Innes Centre) for his critical reading of this manuscript and Purin Panichphant for his help with figures. This work conducted by the Joint BioEnergy Institute was supported by the Office of Science, Office of Biological and Environmental Research, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.[68]

REFERENCES

(1) Renewable Fuels Association (2011) 2011 Ethanol Industry Outlook, http://www.ethanolrfa.org/pages/annual-industry-outlook.

(2) Zhang, F., Rodriguez, S., and Keasling, J. D. (2011) Metabolic engineering of microbial pathways for advanced biofuels production. *Curr. Opin. Biotechnol.* 22, 775−783.

(3) McEwen, J. T., and Atsumi, S. (2012) Alternative biofuel production in non-natural hosts. *Curr. Opin. Biotechnol. 23*, 744−750.
(4) Hanai, T., Atsumi, S., and Liao, J. C. (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. *Appl. Environ. Microbiol. 73*, 7814−7818.
(5) Jojima, T., Inui, M., and Yukawa, H. (2007) Production of isopropanol by metabolically engineered *Escherichia coli*. *Appl. Environ. Biotechnol. 77*, 1219−1224.
(6) Inokuma, K., Liao, J. C., Okamoto, M., and Hanai, T. (2010) Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. *J. Biosci. Bioeng. 110*, 696−701.
(7) Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J. Y., Hanai, T., and Liao, J. C. (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng. 10*, 305−311.
(8) Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N., and Yukawa, H. (2008) Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. *Appl. Microbiol. Biotechnol. 77*, 1305−1316.
(9) Nielsen, D. R., Leonard, E., Yoon, S.-H., Tseng, H.-C., Yuan, C., and Prather, K. L. J. (2009) Engineering alternative butanol production platforms in heterologous bacteria. *Metab. Eng. 11*, 262−273.
(10) Bond-Watts, B. B., Bellerose, R. J., and Chang, M. C. Y. (2011) Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. *Nat. Chem. Biol. 7*, 222−227.
(11) Shen, C. R., Lan, E. I., Dekishima, Y., Baez, A., Cho, K. M., and Liao, J. C. (2011) Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol. 77*, 2905−2915.
(12) Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., Ouellet, M., and Keasling, J. D. (2008) Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. *Microb. Cell Fact. 7*, 36.
(13) Atsumi, S., Hanai, T., and Liao, J. C. (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature 451*, 86−89.
(14) Baez, A., Cho, K.-M., and Liao, J. C. (2011) High-flux isobutanol production using engineered *Escherichia coli*: a bioreactor study with in situ product removal. *Appl. Microbiol. Biotechnol. 90*, 1681−1690.
(15) Atsumi, S., and Liao, J. C. (2008) Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Appl. Environ. Microbiol. 74*, 7802−7808.
(16) Shen, C. R., and Liao, J. C. (2008) Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. *Metab. Eng. 10*, 312−320.
(17) Atsumi, S., Wu, T.-Y., Machado, I. M. P., Huang, W.-C., Chen, P.-Y., Pellegrini, M., and Liao, J. C. (2010) Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Mol. Syst. Biol. 6*, 449.
(18) Atsumi, S., Wu, T.-Y., Eckl, E.-M., Hawkins, S. D., Buelter, T., and Liao, J. C. (2010) Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Appl. Microbiol. Biotechnol. 85*, 651−657.
(19) Cann, A. F., and Liao, J. C. (2008) Production of 2-methyl-1-butanol in engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol. 81*, 89−98.
(20) Connor, M. R., Cann, A. F., and Liao, J. C. (2010) 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. *Appl. Microbiol. Biotechnol. 86*, 1155−1164.
(21) Zhang, K., Sawaya, M. R., Eisenberg, D. S., and Liao, J. C. (2008) Expanding metabolism for biosynthesis of nonnatural alcohols. *Proc. Natl. Acad. Sci. U.S.A. 105*, 20653−20658.
(22) Smith, K. M., Cho, K.-M., and Liao, J. C. (2010) Engineering *Corynebacterium glutamicum* for isobutanol production. *Appl. Microbiol. Biotechnol. 87*, 1045−1055.
(23) Higashide, W., Li, Y., Yang, Y., and Liao, J. C. (2011) Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl. Environ. Microbiol. 2011*, 2727−2733.
(24) Atsumi, S., Higashide, W., and Liao, J. C. (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol. 27*, 1177−1180.
(25) Huo, Y.-X., Cho, K.-M., Rivera, J. G. L., Monte, E., Shen, C. R., Yan, Y., and Liao, J. C. (2011) Conversion of proteins into biofuels by engineering nitrogen flux. *Nat. Biotechnol. 29*, 346−351.
(26) Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J. L., Molina-Jouve, C., and Nicaud, J. M. (2009) *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res. 48*, 375−387.
(27) Lu, Y. J., Zhang, Y. M., and Rock, C. O. (2004) Product diversity and regulation of type II fatty acid synthases. *Biochem. Cell Biol. 82*, 145−155.
(28) Rock, C. O., and Jackowski, S. (2002) Forty years of bacterial fatty acid synthesis. *Biochem. Biophys. Res. Commun. 292*, 1155−1166.
(29) Schweizer, E., and Hofmann, J. (2004) Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. *Microbiol. Mol. Biol. Rev 68*, 501−517.
(30) Tehlivets, O., Scheuringer, K., and Kohlwein, S. D. (2007) Fatty acid synthesis and elongation in yeast. *Biochim. Biophys. Acta 1771*, 255−270.
(31) Cao, Y., Yang, J., Xian, M., Xu, X., and Liu, W. (2010) Increasing unsaturated fatty acid contents in *Escherichia coli* by coexpression of three different genes. *Appl. Microbiol. Biotechnol. 87*, 271−280.
(32) Liu, T., Vora, H., and Khosla, C. (2010) Quantitative analysis and engineering of fatty acid biosynthesis in *E. coli*. *Metab. Eng. 12*, 378−386.
(33) Lu, X., Vora, H., and Khosla, C. (2008) Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. *Metab. Eng. 10*, 333−339.
(34) US Environmental Protection Agency (2012) 2012 Renewable Fuel Standards, http://www.gpo.gov/fdsys/pkg/FR-2012-01-09/pdf/2011-33451.pdf.
(35) Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl. Acad. Sci. U.S.A. 103*, 11206−11210.
(36) Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S. B., and Keasling, J. D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature 463*, 559−562.
(37) Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., Soon Lee, T., Tullman-Ercek, D., Voigt, C. A., Simmons, B. A., and Keasling, J. D. (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 108*, 19949−19954.
(38) Johnson, B., Nelson, S. J., and Brown, C. M. (1972) Influence of glucose concentration on the physiology and lipid composition of some yeasts. *Antonie van Leeuwenhoek 38*, 129−136.
(39) Kalscheuer, R., Luftmann, H., and Steinbuchel, A. (2004) Synthesis of novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. *Appl. Environ. Microbiol. 70*, 7119−7125.
(40) Zhang, F., Carothers, J. M., and Keasling, J. D. (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol. 30*, 354−359.
(41) Dellamonaco, C., Clomburg, J. M., Miller, E. N., and Gonzalez, R. (2011) Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. *Nature 476*, 355−359.
(42) Goh, E.-B., Baidoo, E. E. K., Keasling, J. D., and Beller, H. R. (2012) Engineering of bacterial methyl ketone synthesis for biofuels. *Appl. Environ. Microbiol. 78*, 70−80.
(43) Schirmer, A., Rude, M. A., Li, X., Popova, E., and del Cardayre, S. B. (2010) Microbial biosynthesis of alkanes. *Science 329*, 559−562.
(44) Das, D., Eser, B. E., Han, J., Sciore, A., and Marsh, E. N. G. (2011) Oxygen-independent decarbonylation of aldehydes by cyanobacteria aldehyde decarbonylase: a new reactino of diiron enzymes. *Angew. Chem., Int. Ed. 50*, 7148−7152.
(45) Eser, B. E., Das, D., Han, J., Jones, P. R., and Marsh, E. N. G. (2011) Oxygen-independent alkane formation by non-heme iron-

dependent cyanobacterial aldehyde decarbonylase: investigation of kinetics and requirement for an external electron donor. *Biochemistry 50*, 10743−10750.
(46) Li, N., Nørgaard, H., Warui, D. M., Booker, S. J., Krebs, C., and Bollinger, J. M., Jr. (2011) Conversion of fatty aldehydes to alka(e)nes and formate by a cyanobacteria aldehyde decarbonylase: cryptic redox by an unusual dimetal oxygenase. *J. Am. Chem. Soc. 133*, 6158−6161.
(47) Beller, H. R., Goh, E.-B., and Keasling, J. D. (2010) Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*. *Appl. Environ. Microbiol. 76*, 1212−1223.
(48) Rude, M. A., Baron, T. S., Brubaker, S., Alibhai, M., del Cardayre, S. B., and Schirmer, A. (2011) Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase from *Jeotgalicoccus* species. *Appl. Environ. Microbiol. 77*, 1718−1727.
(49) Wang, W., Li, J., Wang, K., Huang, C., Zhang, Y., and Oldfied, E. (2010) Organometallic mechanism of action and inhibition of the 4Fe-4S isoprenoid biosynthesis protein GcpE (IspG). *Proc. Natl. Acad. Sci. U.S.A. 107*, 11189−11193.
(50) Wang, W., Wang, K., Liu, Y.-L., No, J.-H., Li, J., Nilges, M. J., and Oldfied, E. (2010) Bioorganometallic mechanism of action, and inhibition, of IspH. *Proc. Natl. Acad. Sci. U.S.A. 107*, 4522−4527.
(51) Wang, W., Wang, K., Span, I., Jauch, J., Bacher, A., Groll, M., and Oldfied, E. (2012) Are free radicals involved in IspH catalysis? An EPR and crystallographic investigation. *J. Am. Chem. Soc. 134*, 11225−11234.
(52) Withers, S. T., Gottlieb, S. S., Lieu, B., Newman, J. D., and Keasling, J. D. (2007) Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. *Appl. Environ. Microbiol. 73*, 6277−6283.
(53) Wang, C., Kim, J.-Y., Choi, E.-S., and Kim, S.-W. (2011) Microbial production of farnesol (FOH): Current states and beyond. *Process Biochem. 46*, 1221−1229.
(54) Song, L. (2006) A soluble form of phosphatase in *Saccharomyces cerevisiae* capable of converting farnesyl diphosphate into *E,E*-farnesol. *Appl. Biochem. Biotechnol. 128*, 149−157.
(55) Ohto, C., Muramatsu, M., Obata, S., Sakuradani, E., and Shimizu, S. (2009) Overexpression of the gene encoding HMG-CoA reductase in *Saccharomyces cerevisiae* for production of prenyl alcohols. *Appl. Microbiol. Biotechnol. 82*, 837−845.
(56) Asadollahi, M. A., Maury, J., Møller, K., Nielsen, K. F., Schalk, M., Clark, A., and Nielsen, J. (2008) Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: Effect of *ERG9* repression on sequiterpene biosynthesis. *Biotechnol. Bioeng. 99*, 666−677.
(57) Wang, C., Yoon, S.-H., Shah, A. A., Chung, Y.-R., Kim, J.-Y., Choi, E.-S., Keasling, J. D., and Kim, S.-W. (2010) Farnesol production from *Escherichia coli* by harnessing the exogenous mevalonate pathway. *Biotechnol. Bioeng. 107*, 421−429.
(58) Avé, D. A., Gregory, P., and Tingey, W. M. (1987) Aphid repellent sesquiterpenes in glandular trichomes of *Solanum berthaultii* and *S. tuberosum*. *Entomol. Exp. Appl. 44*, 131−138.
(59) Šobotník, J., Hanus, R., Kalinová, B., Piskorski, R., Cvačka, J., Bourguignon, T., and Roisin, Y. (2008) (*E,E*)-α-Farnesene, an alarm pheromone of the terminte *Prorhinotermes canalifrons*. *J. Chem. Ecol. 34*, 478−486.
(60) Renninger, N. S., McPhee, D. J. (2008) Fuel compositions comprising farnesane and farnesane derivatives and method of making and using same, US patent 7399323.
(61) Rude, M. A., and Schirmer, A. (2009) New microbial fuels: a biotech perspective. *Curr. Opin. Microbiol. 12*, 274−281.
(62) Harvey, B. G., Wright, M. E., and Quintana, R. L. (2010) High-density renewable fuels based on the selective dimerization of pinenes. *Energy Fuels 24*, 267−273.
(63) Reiling, K. K., Yoshikuni, Y., Martin, V. J. J., Newman, J., Bohlmann, J., and Keasling, J. D. (2004) Mono and diterpene production in *Escherichia coli*. *Biotechnol. Bioeng. 87*, 200−212.
(64) Ignea, C., Cvetkovic, I., Loupassaki, S., Kefalas, P., Johnson, C. B., Kampranis, S. C., and Makris, A. M. (2011) Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. *Microb. Cell Fact. 10*, 4.
(65) Ryder, J. A. (2009) Jet fuel compositions, U.S. patent 7589243.
(66) Carter, O. A., Peters, R. J., and Croteau, R. (2003) Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry 64*, 425−433.
(67) Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., and Lee, T. S. (2011) Identification and microbial production of a terpene-based advanced biofuel. *Nat. Commun. 2*, 483.
(68) McAndrew, R. P., Peralta-Yahya, P. P., DeGiovanni, A., Pereira, J. H., Hadi, M. Z., Keasling, J. D., and Adams, P. D. (2011) Structure of a three-domain sesquiterpene synthase: A prospective target for advanced biofuels production. *Structure 19*, 1876−1884.
(69) Martin, V. J. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol. 21*, 796−802.
(70) Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature 440*, 940−943.
(71) Pitera, D. J., Paddon, C. J., Newman, J. D., and Keasling, J. D. (2006) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab. Eng. 9*, 193−207.
(72) Farmer, W. R., and Liao, J. C. (2001) Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog. 17*, 57−61.
(73) Kim, S.-W., and Keasling, J. D. (2001) Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng. 72*, 408−415.
(74) Alper, H., Fischer, C., Nevoigt, E., and Stephanopoulos, G. (2005) Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A. 102*, 12678−12683.
(75) Alper, H., Jin, Y.-S., Moxley, J. F., and Stephanopoulos, G. (2005) Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng. 7*, 155−164.
(76) Alper, H., Miyaoku, K., and Stephanopoulos, G. (2005) Construction of lycopene-overprducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. *Nat. Biotechnol. 23*, 612−616.
(77) Farmer, W. R., and Liao, J. C. (2000) Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat. Biotechnol. 18*, 533−537.
(78) Oldiges, M., Lütz, S., Pflug, S., Schroer, K., Stein, N., and Wiendahl, C. (2007) Metabolomics: current state and evolving methodologies and tools. *Appl. Microbiol. Biotechnol. 76*, 495−511.
(79) Aebersold, R., and Mann, M. (2003) Mass spectroscopy-based proteomics. *Nature 422*, 198−207.
(80) Khan, M. F., Bennett, M. J., Jumpber, C. C., Percy, A. J., Silva, L. P., and Schriemer, D. C. (2011) Proteomics by mass spectrometry—Go big or go home? *J. Pharmaceut. Biomed. 55*, 832−841.
(81) Picotti, P., Bodenmiller, B., Mueller, L. N., Domon, B., and Aebersold, R. (2009) Full dynamic range proteome anaylsis of *S. cerevisiae* by targeted proteomics. *Cell 138*, 795−806.
(82) Redding-Johanson, A. M., Batth, T. S., Chan, R., Krupa, R., Szmidt, H. L., Adams, P. D., Keasling, J. D., Lee, T. S., Mukhopadhyay, A., and Petzold, C. J. (2011) Targeted proteomics for metabolic pathway optimization: Application of terpene production. *Metab. Eng. 13*, 194−203.
(83) Singh, P., Batth, T. S., Juminaga, D., Dahl, R. H., Keasling, J. D., Adams, P. D., and Petzold, C. J. (2012) Application of targeted proteomics to metabolically engineered *Escherichia coli*. *Proteomics 12*, 1289−1299.
(84) Tyo, K. E. J., Kocharin, K., and Nielsen, J. (2010) Toward design-based engineering of industrial microbes. *Curr. Opin. Microbiol. 13*, 255−262.
(85) Kim, T. Y., Sohn, S. B., Kim, Y. B., Kim, W. J., and Lee, S. Y. (2012) Recent advances in reconstruction and applications of genome-scale metabolic models. *Curr. Opin. Biotechnol. 23*, 617−623.

(86) Medema, M. H., van Raaphorst, R., Takano, E., and Breitling, R. (2012) Computational tools for the synthetic design of biochemical pathways. *Nat. Rev. Microbiol. 10*, 191−202.
(87) Bro, C., Regenberg, B., Förster, J., and Nielsen, J. (2006) In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. *Metab. Eng. 8*, 102−111.
(88) Patil, K. R., Rocha, I., Förster, J., and Nielsen, J. (2005) Evolutionary programming as a platform for *in silico* metabolic engineering. *BMC Bioinf. 6*, 308.
(89) Asadollahi, M. A., Maury, J., Patil, K. R., Schalk, M., Clark, A., and Nielsen, J. (2009) Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through *in silico* driven metabolic engineering. *Metab. Eng. 11*, 328−334.
(90) Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Tradwick, J. D., Osterhout, R. E., Stephen, R., Estadilla, J., Teisen, S., Schreyer, H. B., Andrae, S., Yang, T. H., Lee, S. Y., Burk, M. J., and Van Dien, S. (2011) Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol. 7*, 445−452.
(91) Reed, J. L., Vo, T. D., Schilling, C. H., and Palsson, B. O. (2003) An expanded genome-scale model of Escherichia coli K-12 (iJR904 GSM/GPR). *Genome Biol. 4*, R54.
(92) Cho, A., Yun, H., Park, J. H., Lee, S. Y., and Park, S. (2010) Prediction of novel synthetic pathways for the production of desired chemicals. *BMC Syst. Biol. 4*, 35.
(93) Dunlop, M. J., Dossani, Z. Y., Szmidt, H. L., Chu, H. C., Lee, T. S., Keasling, J. D., Hadi, M. Z., and Mukhopadhyay, A. (2011) Engineering microbial biofuel tolerance and export using efflux pumps. *Mol. Syst. Biol. 7*, 487.
(94) Jensen, P. R., and Hammer, K. (1998) The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl. Environ. Microbiol. 64*, 82−87.
(95) Schlabach, M. R., Hu, J., Li, M., and Elledge, S. J. (2010) Synthetic design of strong promoters. *Proc. Natl. Acad. Sci. U.S.A. 107*, 2538−2543.
(96) Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol. 27*, 946−950.
(97) Carothers, J. M., Goler, J. A., Juminaga, D., and Keasling, J. D. (2011) Model-driven engineering of RNA devices to quantitatively program gene expression. *Science 334*, 1716−1719.
(98) Romero, P. A., and Arnold, F. H. (2009) Exploring protein fitness landscapes by directed evolution. *Nat. Rev. Mol. Cell Biol. 10*, 866−876.
(99) Jäckel, C., and Hilvert, D. (2009) Biocatalysts by evolution. *Curr. Opin. Biotechnol. 21*, 753−759.
(100) Turner, N. J. (2009) Directed evolution drives the next generation of biocatalysts. *Nat. Chem. Biol. 5*, 567−573.
(101) Bastian, S., Liu, X., Meyerowitz, J. T., Snow, C. D., Chen, M. M. Y., and Arnold, F. H. (2011) Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli*. *Metab. Eng. 13*, 345−352.
(102) Zhou, Y., Gao, W., Rong, Q., Jin, G., Chu, H. C., Liu, W., Yang, W., Zhu, Z., Li, G., Zhu, G., Huang, L., and Zhao, Z. K. (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. *J. Am. Chem. Soc. 134*, 3234−3241.
(103) Tokuhiro, K., Muramatsu, M., Ohto, C., Kawaguchi, T., Obata, S., Muramoto, N., Hirai, M., Takahashi, H., Kondo, A., Sakuradani, E., and Shimizu, S. (2009) Overproduction of geranylgeraniol by metabolically engineered *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol. 75*, 5536−5543.
(104) Albertsen, L., Chen, Y., Bach, L. S., Rattleff, S., Maury, J., Brix, S., Nielsen, J., and Mortensen, U. H. (2011) Diversion of flux toward sesquiterpene production in *Saccharomyces cerevisiae* by fusion of host and heterologous enzymes. *Appl. Environ. Microbiol. 77*, 1033−1040.
(105) Dueber, J. E., Wu, G. C., Malmirchegini, G. R., Moon, T. S., Petzold, C. J., Ullal, A. V., Prather, K. L. J., and Keasling, J. D. (2009) Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol. 27*, 753−759.