JBEI Performance Metric for FY16: Develop an improved metabolic engineering method for modifying microorganisms for biofuel production from cellulosic sugars.

Q1 Metric: New metabolic pathways developed at JBEI for microbial conversion of cellulosic sugars to biofuels.

1. BACKGROUND

JBEI's Fuel Synthesis Division has targeted advanced biofuels that have favorable fuel properties and are compatible with existing engine design and fuel distribution infrastructure. Selection of fuel targets has resulted in part from interactions with the Combustion Research Facility at Sandia National Laboratories. Design of metabolic pathways for biofuels has leveraged existing pathways to the extent possible (e.g., isoprenoid biosynthesis, fatty acid biosynthesis and β-oxidation, polyketide synthases) with additional catalytic capabilities engineered to optimally produce the fuel target. In this report, we describe development of some new fuels at JBEI with an emphasis on recently published work.

2. FATTY ACID-DERIVED BIOFUELS

Fatty acid biosynthesis is a widely distributed and essential metabolic capability found throughout the Bacterial and Eukarya domains of life. As such, fatty acids are readily available, potentially renewable compounds that have been targeted by the biofuel industry because they include highly reduced, aliphatic moieties that mimic the high-energy-density properties that characterize hydrocarbon components of petroleum-derived fuels. However, the carboxylic acid moiety has characteristics that are not compatible with model fuels (such as polarity, reactivity, and O content) and needs to be modified for use in biofuels, for example by esterification, reduction, or decarboxylation. Accelerated research-and-development activity in biofuels over the past decade has facilitated the discovery of a variety of enzymes and metabolic pathways that enable biochemical conversion of fatty acids (and intermediates of fatty acid biosynthesis) to a range of industrially relevant compound classes, such as fatty acid alkyl esters, fatty alcohols, aliphatic methyl ketones, alkanes, and alkenes (α-olefins and long-chain internal alkenes), as has been described in recent reviews (e.g., Beller et al., 2015)).

2.1 Previous work: fatty acid ethyl esters (FAEE) and long-chain alkenes

This report will focus on recent research, but here we summarize some previous JBEI work with fatty acid-derived products, specifically, development of pathways for fatty acid ethyl esters (FAEE) and long-chain alkenes (Figure 1). For a given chain length and degree of unsaturation, FAEE have similar fuel properties to fatty acid methyl esters (FAME), which are the components of the widely used "biodiesel" fuel, made by chemical transesterification of plant- or animal-derived triacylglycerols. Steen and co-workers engineered *E. coli* to produce FAEE *in vivo* with glucose as the sole carbon source (Steen et al., 2010). To produce FAEE, *E. coli* has been engineered to carry out multiple metabolic reactions: (1) production of ethanol by heterologous expression of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Zymomonas mobilis*, (2) production of acyl-CoAs *via* overexpression of a modified, cytoplasmically directed thioesterase ('*tesA*) and a native acyl-CoA synthetase (*fadD*), and (3) esterification of the ethanol and acyl-CoAs with a heterologously expressed acyltransferase (wax ester synthase/acyl-CoA:diacylglycerol acyltransferase, or WS/DGAT) encoded by *atfA* from *Acinetobacter baylyi* strain ADP1. A substantial improvement in FAEE production in *E. coli* was achieved by introducing a dynamic sensor-regulator system (DSRS) focusing on the acyl-

CoA-responsive transcription factor, FadR (Zhang et al., 2012). The DSRS resulted in a FAEE titer of 1.5 g/L (batch incubation with 2% glucose), representing a reported 28% of maximum theoretical yield, which is the best performance reported for FAEE to date.

In other research on new fatty acid-related pathways, we discovered the enzymes needed for long-chain alkene biosynthesis by head-to-head condensation of fatty acids (or more specifically, acyl-CoAs) in the actinobacterium *Micrococcus luteus*. This research followed historical work from the 1960s, in which two research groups reported that *Sarcina lutea* ATCC 533 (now *Kocuria rhizophila*) synthesized *iso*- and *anteiso*-branched, long-chain (primarily C_{25} to C_{29}) alkenes by an unknown enzymatic mechanism (Albro and Dittmer, 1969a; Albro and Dittmer, 1969b; Tornabene et al., 1967). In 2010 (Beller et al., 2010), we identified OleABCD as being essential for long-chain alkene biosynthesis in *Micrococcus luteus* ATCC 4698, a close relative of *S. lutea*. Heterologous expression of *M. luteus oleABCD* in *E. coli* resulted in production of C_{27} and C_{29} alkenes, whereas expression of *oleA* alone resulted in unsaturated aliphatic monoketones (primarily C_{27}) rather than alkenes. *In vitro* studies with purified recombinant OleA and myristoyl-CoA (14:0) resulted in the same C_{27} monoketone that was observed during *in vivo* studies with *oleA*. C_{27} and C_{29} alkenes are too long to serve as diesel fuels, as they are solids at room temperature, but they could be used as feedstocks for cracking to shorter hydrocarbons suitable for use as fuels.

Figure 1. Generic structures for FAEE (left) and long-chain alkenes (right) produced at JBEI.

2.2 **Methyl ketones**

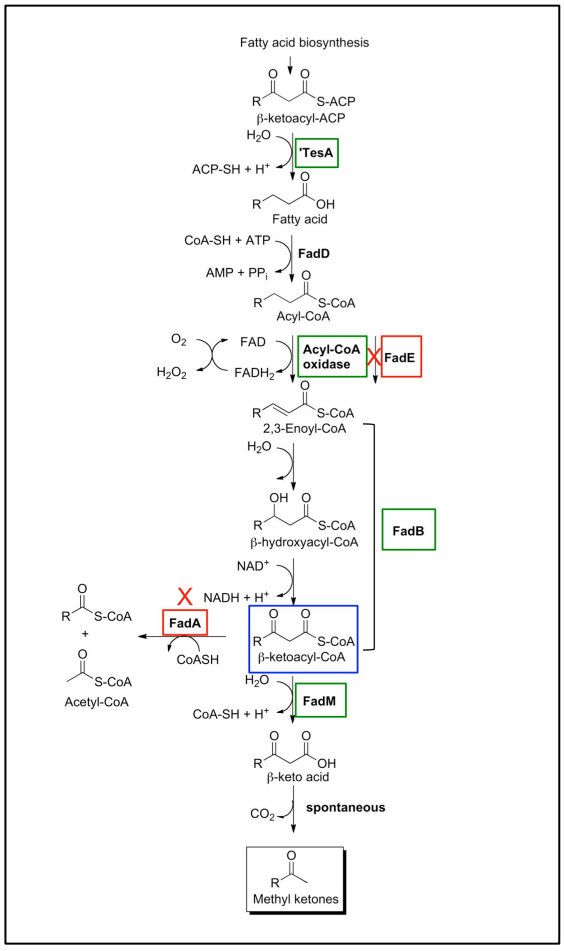
2.2.1 *Background and rationale for selection*

Aliphatic methyl ketones are naturally occurring compounds that were first discovered in rue (*Ruta graveolens*) more than a century ago and have since been commonly found in microorganisms, plants, insects, and mammalian cells. These compounds, such as 2-undecanone (C_{11}) and 2-tridecanone (C_{13}), have a variety of important natural and commercial roles, including acting as pheromones and natural insecticides in plants, providing scents in essential oils and flavoring in cheese and other dairy products, use as commercial insect repellents, and potentially serving as diesel fuel blending agents, as we have proposed (Goh et al. 2012). We have determined the cetane number for 2-undecanone as 56.6 and for a 50/50 (wt/wt) mixture of 2-undecanone and 2-tridecanone as 58.4; these values compare favorably to cetane numbers for diesel fuel marketed in the U.S., which typically range between 40 and 45.

2.2.2 *Pathway development*

We have engineered a novel metabolic pathway in *Escherichia coli* to overproduce saturated and monounsaturated aliphatic methyl ketones in the C_{11} to C_{17} (diesel) range. The host was a fatty acid-overproducing *E. coli* strain that overexpressed a truncated, cytoplasmically directed version of a native thioesterase (‘*tesA*) and had a chromosomal deletion of the acyl-CoA dehydrogenase *fadE*. Features of the first generation of methyl ketone-overproducing *E. coli* (Goh et al., 2012) included the following (Figure 2): (a) overproduction of β-ketoacyl-coenzyme A (CoA) thioesters achieved by modification of the β-oxidation pathway (specifically, overexpression of a heterologous acyl-CoA oxidase from *Micrococcus luteus* and native FadB, and chromosomal deletion of *fadA*) and (b) overexpression of a native thioesterase (FadM). FadM was previously associated with oleic acid degradation, not methyl ketone synthesis, but

overexpression of this thioesterase enhanced methyl ketone titers ca. 10-fold. Whole-genome transcriptional studies of modified fatty acid-overproducing strains led to the discovery that FadM is a valuable catalyst for enhancing methyl ketone production (Goh et al., 2012).



We have transferred the methyl ketone pathway into *Ralstonia eutropha*, a bacterium that can grow chemolithoautotrophically with CO_2 and H_2 as the sole carbon source and electron donor, respectively (Muller et al., 2013). *R. eutropha* was reported to produce up to 180 mg/L of methyl ketones under such chemolitho-autotrophic growth conditions.

Figure 2. Summary of first-generation engineered pathway to convert fatty acids to methyl ketones in *E. coli* DH1. Green boxes indicate overexpressed genes and red boxes indicate chromosomal deletions. The blue box indicates the putative substrate for FadM (producing free β-keto acids). The final step, decarboxylation of β-keto acids to form methyl ketones, is shown as "spontaneous"; this has been demonstrated by *in vitro* reconstitution studies (Goh et al., 2014).

2.2.3 *Optimization*

Recently, we have made genetic modifications to the first-generation *E. coli* strain that improved methyl ketone titer and yield 160-fold (Goh et al., 2014). Host and pathway modifications included balancing overexpression of *fadR* and *fadD* to increase fatty acid flux into the pathway, consolidation of the pathway from two plasmids into one, codon optimization, and knocking out key acetate production pathways (*poxB*, *ackA*, and *pta*) (Figure 3). In our best strain to date, a titer of 1.4 g/L of C_{11} to C_{17} methyl ketones was produced in minimal growth medium with 1% glucose, representing 40% of maximum theoretical yield, and 3.4 g/L methyl ketones was produced after 45 hr of fed-batch fermentation with glucose. These titer and yield values are among the highest reported for any fatty acid-derived fuels or bio-based chemicals.

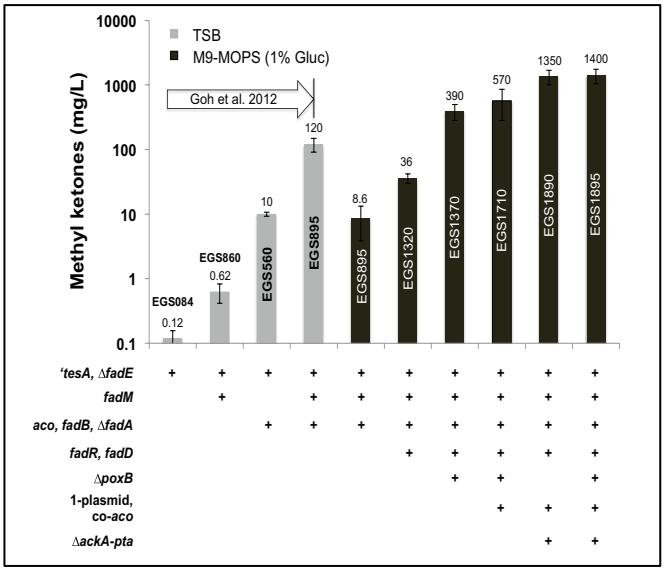


Figure 3. Summary of improvements in performance resulting from engineering modifications to *E. coli* strains bearing the methyl ketone biosynthesis pathway. More details are given by Goh et al. (2014).

3. **ISOPRENOID-DERIVED BIOFUELS**

Isoprenoids have a number of structural attributes that give them favorable fuel properties. For example, their carbon chain lengths (C_5, C_{10}, and C_{15}) are appropriate for a range of transportation fuel types, and their methyl branching, which is a common structural feature of isoprenoids, lowers the freezing point significantly and endows them with excellent cold weather properties as fuels. In recent years, several isoprenoids have been developed and tested as potential gasoline, diesel, and jet fuels with favorable energy contents, cold weather properties, and octane/cetane numbers (reviewed in George et al., 2015). At JBEI, we have engineered isoprenoid biosynthetic pathways to achieve microbial production of biofuels; the pathways and products are summarized in Figure 4.

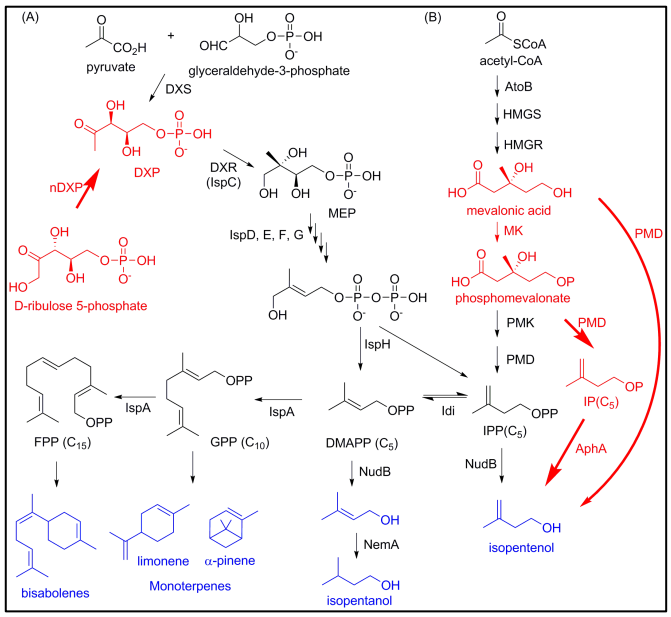


Figure 4. Pathways for isoprenoid-based biofuels and examples of JBEI isoprenoid biofuels. (A) MEP (or DXP) pathway with a variant (nDXP) pathway for DXP from D-ribulose 5-phosphate. (B) MVA pathway with variant pathways for isopentenol. Biofuel products we discuss here (in blue) are bisabolene (C_{15}), limonene (C_{10}), pinene (C_{10}), isopentanol (C_5), and isopentenol (C_5). New novel isoprenoid pathways we have invented at JBEI are shown in red with bold arrows.

3.1 **Background of the isoprenoid-producing pathways**

Isoprenoid biosynthesis is initiated by the formation of two universal C_5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Prenyltransferase enzymes condense IPP to prenyl diphosphates of various lengths, and prenyl diphosphates are the substrates of terpene synthases, a class of enzymes that the formation of various terpenes. The starting C_5 precursors can be synthesized by two routes: the 2-methyl-D-erythritol-4-phosphate (MEP) or 1-deoxy-D-xylulose 5-phosphate (DXP) pathway and the mevalonate (MVA) pathway (Figure 4). Between these two pathways, we have primarily engineered the MVA pathway, which is not endogenous in the microbial production host *E. coli*. Although the MVA pathway is energetically less efficient than the native MEP pathway, implementing a heterologous MVA pathway in bacterial hosts is advantageous due to its lack of endogenous regulation.

The functional expression of the MVA pathway enzymes in a heterologous host has been achieved previously (Martin et al., 2003). We have exploited synthetic biology to allow this heterologous pathway to work efficiently in new hosts, and also employed multiple omics tools to monitor and optimize the pathway enzymes and metabolites to improve yields and titers of isoprenoid production (Alonso-Gutierrez et al., 2015; George et al., 2014).

For application to biofuel production, we need to search for adequate fuel targets and to employ appropriate terpene synthases that are capable of producing these fuel molecules from

the corresponding prenyl diphosphate precursors. Terpene synthases are derived from various sources including plants, fungi, and bacteria. We have bioprospected various terpene synthases for their activity in a microbial host, and also optimized the enzyme activities for improved terpene production. In the following section, we report various isoprenoid biofuels that we have developed at JBEI and the metabolic engineering efforts we have implemented in the past few years.

3.2 **Isoprenoid biofuels and pathway engineering efforts**

3.2.1 *Bisabolene*

The hydrogenated C_{15} isoprenoids such as bisabolane and farnesane have cetane numbers in the expected range for diesel fuels (40~60) and display better cold-temperature properties than those of D2 diesel (e.g., freezing point of -78°C for bisabolane vs -3°C for D2 diesel). Furthermore, the cyclic structure of bisabolane contributes to increase the density of the fuel (0.88 g/mL), which will also increase its volumetric energy density. Plants are the natural source of bisabolene, but the production from plants is quite low and it would be inefficient to retrieve the product for use as a biofuel. Therefore, engineered microbial platforms may be the most convenient and cost-effective means to produce bisabolene (Peralta-Yahya et al., 2011). Using the same biosynthetic platform as has been used to produce the anti-malarial drug artemisinin, we bioprospected various bisabolene synthases from plants and discovered that the synthase from *Abies grandis* has good and specific activity for bisabolene production. We further engineered the pathway to improve the bisabolene yield both in *E. coli* and yeast. Using a proteomics-based metabolic engineering method, we achieved 1.2 g/L of bisabolene production in batch culture, which is 35% of the maximum theoretical yield (Alonso-Gutierrez et al., 2015).

Terpene synthase activity is one of the limiting factors in bisabolene synthesis and we developed a screening method for more active terpene synthase mutants. Bisabolene was found to protect yeast against the disruptive action of nonionic surfactants such as Tween 20 (T20), and the strain with more active bisabolene synthase could survive in the presence of higher T20 concentrations. Cultures grown in the presence of T20 for 14 days produced bisabolene at titers up to 4-fold higher than cultures grown with an overlay of dodecane, and 20-fold higher than cultures grown without dodecane (Kirby et al., 2014).

3.2.2 *Monoterpenes – limonene and pinene*

Monoterpenes are produced from geranyl diphosphate (GPP, C_{10}) by various monoterpene synthases, and have been used as ingredients in cosmetics, food flavorings, cleaning products, and drugs. Among these monoterpenes, the hydrogenated products of mycene and ocimene (2,6-dimethyloctane) and the hydroxylated compounds such as linalool and geraniol have been suggested as potential biofuel precursors. Recently, hydrogenated dimers of α- and β-pinene have been synthesized chemically and showed a high energy density similar to that of JP-10 (Harvey et al., 2010).

By employing the MVA pathway, we achieved high-titer production of the monoterpene limonene in *E. coli*. A step-by-step optimization of limonene production was performed in *E. coli* strains with the heterologous MVA pathway, GPP synthase (GPPS) from *Arabidopsis thaliana*, and limonene synthase from *Mentha spicata* (Alonso-Gutierrez et al., 2013). Then a targeted proteomics approach was used to determine the enzyme level under various expression conditions to understand the correlation of production and pathway-enzyme levels. To analyze this multivariate proteomics data, we applied Principal Component Analysis (PCA) to a collection of proteomics data for limonene pathway enzymes, and we plotted these data with

target molecule production data to pinpoint specific enzymes that need to have their expression level adjusted to balance the pathway and maximize biofuel production. PCA of Proteomic data (PCAP)-guided metabolic pathway engineering resulted in over a 30% titer improvement to 650 mg/L of limonene in a batch culture (Alonso-Gutierrez et al., 2015).

Microbial production of pinene was also demonstrated in *E. coli* with relatively low titer (Sarria et al., 2014). We combinatorially screened various pinene synthases (PSs) and GPPS enzymes to improve flux through the last two steps of the pathway. By combining expression of three PS and three GPPS from conifers, we achieved about 28 mg/L of pinene using GPPS and PS from *Abies grandis*, and we designed GPPS-PS protein fusions to reduce GPP product inhibition and toxicity by substrate channeling, producing 32.4 mg/L of pinene.

3.2.3 *Isopentenol/isopentanol*

Branched C_5 alcohols [isopentanol (3-methyl-1-butanol) and isopentenols (3-methyl-3-butenol and 3-methyl-2-butenol)] have higher energy contents than ethanol and high octane numbers (RON, or research octane number, of 92-102), which supports their use as gasoline replacements and as anti-knock additives (Mack et al., 2014). They have also been tested in various engine types and have better gasoline-like properties than ethanol [collaboration with Combustion Research Facility (CRF) at Sandia National Laboratory]. Isopentenols were produced in engineered *E. coli* using a heterologous MVA pathway. Searches for an enzyme that hydrolyzes IPP and DMAPP to make isopentenols identified NudF (*Bacillus subtilis*) and NudB (*E. coli*) and these genes were used for high-titer production of isopentenols (Chou and Keasling, 2012; Withers et al., 2007).

A more systematic approach using targeted proteomics and metabolomics led to higher isopentenol yield and titer. We have analyzed proteomics data together with growth rates, titers, and key metabolite levels from microbial growth experiments. The omics data and the correlation analysis improved understanding of pathway dynamics and facilitated high-titer isopentenol production, and provided guidance on metabolic engineering approaches to improve biofuel production (George et al., 2014). Using this approach, a titer of 2.2 g isopentenol /L (up to 70% of apparent theoretical yield) was achieved in *E. coli* (George et al., 2015).

Two limitations of using the MVA pathway are the toxicity of the universal intermediate IPP and a high demand for ATP to produce diphosphate precursors. To overcome limitations intrinsic to IPP accumulation and to relieve "unnecessary" consumption of ATPs for isopentenol production, we designed "IPP-bypass" pathways for isopentenol production (Figure 4). By implementing two previously unidentified promiscuous activities of phosphomevalonate decarboxylase (PMD) and endogenous phosphatase (AphA), we demonstrated that considerable isopentenol titers could be achieved *via* new pathway without producing IPP (Kang et al., 2015). The IPP-bypass pathway was shown to be a robust alternative to the original MVA pathway for isopentenol production, and reduced the engineering burden to balance the upstream MVA pathway and IPP toxicity. Most significantly, the IPP-bypass pathway requires less ATP than the original MVA pathway and was more competitive when aeration was limited, which would significantly reduce operational costs for aeration in a large-scale fermentation.

3.2.4 *MEP (or DXP) pathway engineering*

Unlike *E. coli*, yeast does not have the MEP (or DXP) pathway natively, and functional expression of the MEP pathway in yeast has been addressed as a grand challenge by multiple research groups. At JBEI, we have developed a partially functional MEP pathway in yeast. Starting from a zero-flux pathway, selection of an appropriate IspG enabled detection of MEP-derived ergosterol at levels below 1%. Following this, modifications to the growth conditions

and metabolomics-guided pathway optimization increased the MEP pathway flux to over 33%, close to levels that will support growth in the absence of the native MVA pathway. Using JGI-synthesized plasmids, we are currently working towards improvement of the MEP pathway flux to the point where it can support ergosterol production in the absence of the native MVA pathway.

In *E. coli*, a hypothetical novel route from a pentose phosphate to DXP could enable a more direct pathway from C5 sugars to terpenes and also circumvent regulatory mechanisms that control Dxs. However, there is no enzyme known that can convert a sugar into its 1-deoxy equivalent, and using a *dxs* knockout strain of *E. coli*, we have discovered two alternative enzymes, both of which convert ribulose 5-phosphate directly to DXP as shown in Figure 4 (Kirby et al., 2015). We have demonstrated utility of these genes for engineering isoprenoid production in *E. coli* and further improved flux by fusing the novel DXP proteins (nDXP) to DXP reductase (Dxr), the second enzyme in the DXP pathway (Figure 4). Expression of a Dxr-RibB(G108S) fusion improved bisabolene titers more than 4-fold and alleviated accumulation of intracellular DXP.

4. **POLYKETIDE-DERIVED BIOFUELS**

Polyketides are some of the most diverse molecules known. The enzymes that make them, polyketide synthases (PKSs), perform Claisen condensation reactions between a loaded acyl-ACP intermediate and an α-substituted (H, CH_3, C_2H_5, etc.) malonyl extender unit analogous to fatty acid biosynthesis. This condensation is followed by varying degrees of bextender unit analogous to fatty acid biosynthesis. This condensation is followed by varying degrees of es (PKSs), perform Claisen condensation reactions between a commonly by the activity of a thioesterase domain.

Engineered modular polyketide synthases have the potential to be an extraordinarily effective retrosynthesis platform. Native PKSs assemble and tailor simple, readily available cellular acyl-CoAs into large, complex, chiral molecules (Figure 1). By successfully rearranging existing polyketide modules and domains, one could exquisitely control chemical structure from DNA sequence alone. As an example of the diverse biosynthetic potential of PKSs, we have determined that approximately 20 of the roughly 150 commodity chemicals tracked by the petrochemical market information provider ICIS could be produced by mixing and matching naturally occurring polyketide synthase domains (ISIS Chemicals commodity and product finder; URL http://www.icis.com/chemicals/channel-info-finder/). To form these chemicals, engineered polyketide synthases would load acyl-CoAs accessible in *E. coli,* perform a programmed number of extension reactions (as shown in Figure 5), and then release products using previously published mechanisms. However, this potential has only just begun to be realized as the compounds that have been made using engineered PKSs represent a small fraction of the potentially accessible chemical space. We envision a future in which a single design algorithm, using a molecule of interest as input, successfully combines natural PKS sequences to produce the desired molecule.

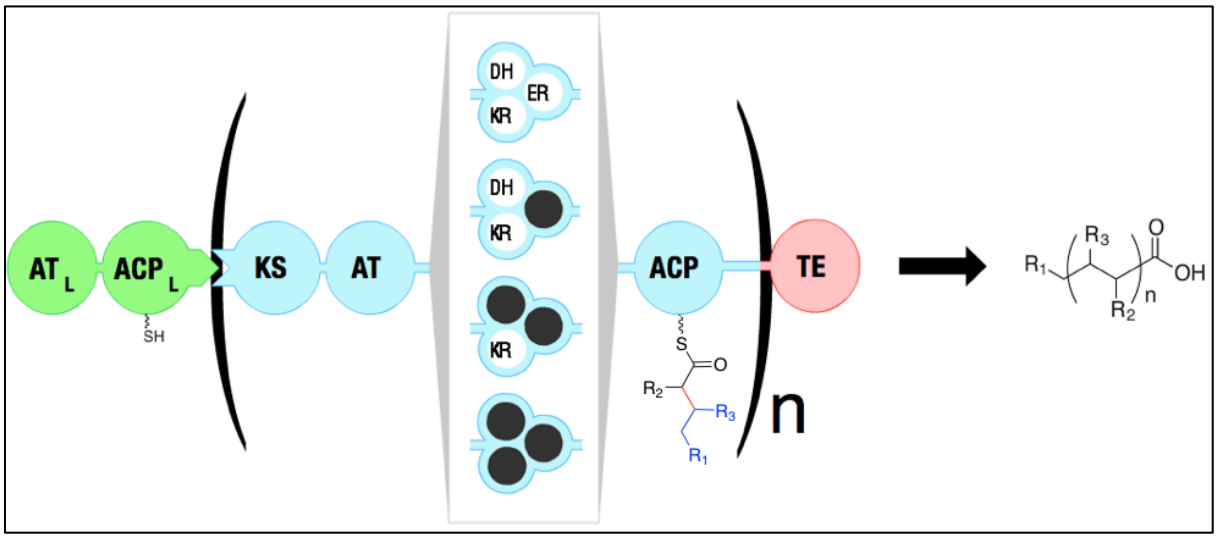


Figure 5. Symbolic representation of a general type I polyketide synthase and a general polyketide product. Biosynthesis begins with the selection of an acyl-CoA by the loading acyl transferase (AT_L) and subsequent transfer to the phosphopantetheine arm of the loading acyl carrier protein (ACP_L). A variable number of extension modules (represented as "n") perform successive elongations of the enzyme-bound intermediates with downstream malonyl or methylmalonyl-ACPs (loaded by their cognate AT domains) via Claisen condensations catalyzed by ketosynthase (KS) domains. Accessory domains often present within a module such as ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains determine the final oxidation state and stereochemistry of the beta-carbonyl attached to the ACP. The bond formed by the KS is represented on the ACP in red; the portion of the extended polyketide chain formed from the malonyl moiety is shown in black; and the portion from the upstream module(s) is shown in blue. In both the general polyketide product and in the intermediate attached to the ACP, R_1 represents the acyl chain from upstream modules or from the loading module and R_2 represents the side-chain on the extender unit, R_3 may be the oxygen of a ketone, an R or S hydroxyl or hydrogen, depending on the number of reducing domains in the particular module employed. Linker–mediated interactions promote chain transfer when modules are contained within different polypeptides. At the end of the synthesis, the final product is released as a free acid or as a lactone by a thioesterase. The bond entirely within the parentheses of the general product may be a double bond if a KR-DH pair is present within a module.

We have begun work on engineering PKSs for production of fuels (Figure 6) and have demonstrated production of 3-hydroxyacids (Yuzawa et al., 2013), MKs (unpublished), EKs (unpublished), and alcohols (unpublished) by constructing hybrid PKSs from parts of various PKS that have the targeted chemistries. The lipomycin PKS (LipPKS) is useful for producing short, highly-branched molecules; the borrelidin PKS (BorA4,5,6) is useful for producing longer molecules that would be useful as diesel or jet fuel. Depending on the final (termination module) on the PKS, it will produce acids, alcohols, alkenes, or ketones. The MKs that we have produced are in the range of molecules useful as gasoline replacements.

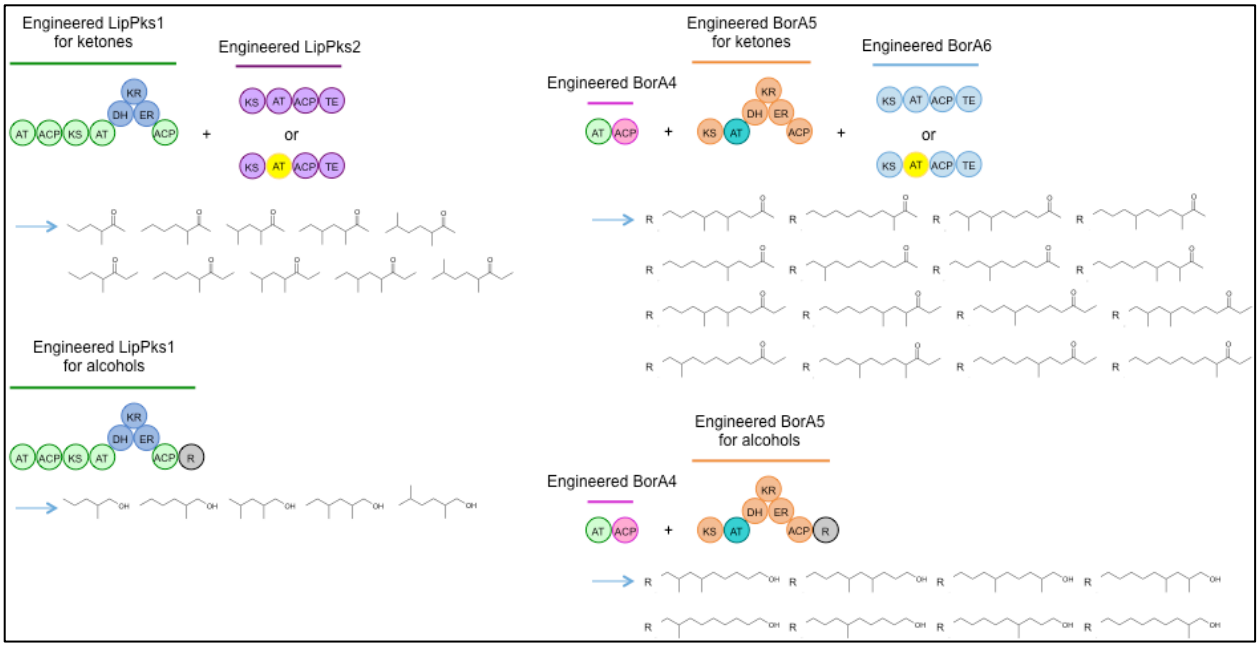


Figure 6. Engineered PKS systems to produce spark ignition fuels (left) and compression ignition fuels (right). Circles of the same color mean that the domain was taken from the same natural source. Light green circles are domains from the LipPks1 module. Purple circles are domains from the LipPks2 module. Pink circles are domains from the BorA4 module. Orange circles are domains from the BorA5 module. Light blue circles are domains from the BorA6 module. Aqua and yellow circles are AT domains that have been swapped to change specificity. Gray circles with an R indicate the reductive domain.

Q1 report Authors: Harry R Beller, Taek Soon Lee, Jay D Keasling.

JBEI Fuel Synthesis Team: Harry R Beller, Taek Soon Lee, Hector Garcia Martin, Nathan J Hillson, Jay D Keasling, Aindrila Mukhopadhyay.

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