Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Leveraging microbial biosynthetic pathways for the generation of 'drop-in' biofuels

Amin Zargar[1,3], Constance B Bailey[1,2], Robert W Haushalter[1,2], Christopher B Eiben[1,2], Leonard Katz[1,3,4] and Jay D Keasling[1,2,3,4,5,6]

Advances in retooling microorganisms have enabled bioproduction of 'drop-in' biofuels, fuels that are compatible with existing spark-ignition, compression-ignition, and gas-turbine engines. As the majority of petroleum consumption in the United States consists of gasoline (47%), diesel fuel and heating oil (21%), and jet fuel (8%), 'drop-in' biofuels that replace these petrochemical sources are particularly attractive. In this review, we discuss the application of aldehyde decarbonylases to produce gasoline substitutes from fatty acid products, a recently crystallized reductase that could hydrogenate jet fuel precursors from terpene synthases, and the exquisite control of polyketide synthases to produce biofuels with desired physical properties (*e.g.*, lower freezing points). With our increased understanding of biosynthetic logic of metabolic pathways, we discuss the unique advantages of fatty acid, terpene, and polyketide synthases for the production of bio-based gasoline, diesel and jet fuel.

Addresses
[1] Joint BioEnergy Institute, Lawrence Berkeley National Laboratory, Emeryville, CA 94608, United States
[2] Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, United States
[3] QB3 Institute, University of California-Berkeley, 5885 Hollis Street, 4th Floor, Emeryville, CA 94608, United States
[4] Synthetic Biology Engineering Research Center, University of California, Berkeley, CA 94720, United States
[5] Department of Chemical & Biomolecular Engineering, Department of Bioengineering, University of California, Berkeley, CA 94720, United States
[6] Novo Nordisk Foundation Center for Biosustainability, Technical University Denmark, DK2970 Horsholm, Denmark

Corresponding author: Keasling, Jay D (jdkeasling@lbl.gov)

Current Opinion in Biotechnology 2017, **45**:156–163

This review comes from a themed issue on **Energy biotechnology**

Edited by **Scott Banta** and **Brian Pfleger**

For a complete overview see the Issue and the Editorial

Available online 17th April 2017

http://dx.doi.org/10.1016/j.copbio.2017.03.004

In 2014, the levels of anthropogenic greenhouse gases (carbon dioxide, methane, and nitrous oxide) reached their highest levels in at least 800 000 years [1,2]. Transportation vehicles contributed approximately 14% of all global greenhouse gas emissions in 2010, with 95% of that derived from petroleum based fuels [3]. If fuels produced from renewable sources such as biomass, landfill gas or atmospheric CO_2 replaced petrochemically-derived fuels, it would reduce CO_2 emissions through the 'closed CO_2 cycle': CO_2 that is burned through combustion is reused from the atmosphere to produce the biofuel [4]. While no current biofuel should not be considered purely carbon neutral after accounting for collateral emissions, the production of 'drop-in' biofuels, fuels that are compatible with existing spark-ignition, compression-ignition and gas-turbine engines, could greatly reduce greenhouse gas emissions [5,6].

Microbial fermentation is a particularly attractive means of producing renewable biofuels. Genetically engineered microbes can utilize feedstocks from non-agricultural sources (*e.g.*, switchgrass) that does not compete with food crops for land mass to produce various fuels and commodity chemicals. In this review, we discuss the microbial production of biofuels derived from three different classes of biosynthetic pathways: fatty acid, isoprenoid, and polyketide. Short chain alcohols (*e.g.*, isobutanol, 1-butanol) are an important class of drop-in fuels, and will continue to be in the future. As they are produced from different pathways and are well reviewed [7], we exclude their discussion here. We provide the maximum theoretical mass yield and highest reported titers for these select 'drop-in' biofuels (Table 1) and discuss the production pathways. Fatty acid biosynthesis is the most well-established pathway to produce biofuels, and we discuss the recent work to synthesize short and medium chain alkanes as constituents of gasoline and diesel. Isoprenoid hydrocarbons often contain branching and ring structures that have high energy content, low water miscibility and reduced premature ignition, rendering them attractive biofuel substitutes for diesel and even jet fuel. Polyketide synthases, although they have been explored less thoroughly for such applications, also have attractive biosynthetic logic to produce high performance biofuels.

Table 1

Maximum possible theoretical yield is given for each compound based on the substrate glucose under anaerobic conditions. The highest reported titres for each type of compound is illustrated

Pathway	Compounds	Mass yield % (g/g_{hexose})	Highest reported titre
Fatty acid synthesis			
	Alkanes (C13–C17)	30.7–30.8%	300 mg/L [11]
	Alkanes (C9–C14)	30.5–30.7%	580 mg/L [16]
	Propane	29.4%	32 mg/L [20••]
Isoprene synthesis			
	Bisabolene	32.4%	5.2 g/L [33]
	Farnesene	32.4%	1.1 g/L [36•]
	Pinene	32.4%	32 mg/L [38]
	Limonene	32.4%	700 mg/L [46]
Polyketide synthesis			
	Multi-methyl-branched esters	27.2–27.6%	98 mg/L [51]
	Pentadecane	30.8%	140 mg/L [52•]

Biofuels derived from fatty acid biosynthetic pathways

While the fatty acid pathway has been leveraged to produce alcohol, ketone, ester and olefin biofuel products [8–10], there is an exciting new avenue to synthesize short chain and medium chain alkanes that could be used in place of gasoline and diesel. Initiated by acetyl-CoA, fatty acid biosynthesis in *Escherichia coli* is performed by the fatty acid synthase complex (FAS) II that uses multiple, discrete enzymes to generate a saturated fatty acid (typically 14–18 carbons in length). Alkanes could be produced from the products of FAS (fatty-acyl carrier protein (ACP), free fatty acid, and fatty-acyl-CoA (Figure 1a) using a fatty aldehyde decarbonylase (ADO), first identified in the cyanobacterium *Synechococcus elongatus*, which converts fatty aldehydes to alkanes [11].

In a seminal report, Schirme *et al.* demonstrated that an acyl carrier protein reductase (AAR) could produce fatty aldehydes directly from fatty acyl-ACPs in *E. coli* (Figure 1b) [11]. With the heterologous expression of an improved ADO from *Nostoc punctiforme*, 300 mg/L of odd-numbered C13–C17 hydrocarbons were produced, 80% present extracellularly. A subsequent report demonstrated an AAR from *Bacillus subtilis* could produce even-numbered C14 and C16 when expressed with ADO [12]. While this method is appropriate for diesel fuel type molecules, the limitations in the chain-length profile of hydrocarbons synthesized precludes producing a substitute for gasoline, which is a blend of short-chain hydrocarbons (typically three to nine carbons) [13].

There are several advantages to producing alkanes from FFAs or acyl-CoA: fatty acids have been produced in higher abundance than fatty acyl-ACPs, production from fatty acids affords better control over chain length, and the pool of fatty acids can be manipulated [9,14,15]. A recent study described the *in vivo* production of a modified acyl-ACP thioesterase to produce short-chain fatty acids, which were then appended to CoA via an overexpressed fatty-acyl-CoA ligase (Figure 1b). The cells produced 580 mg/L of short chain alkanes from fatty-acyl-CoAs by the sequential reaction of *Clostridium acetobutylicum* fatty acyl-CoA reductase (ACR1) and *Arabidopsis thaliana* ADO [16]. As such, this strategy relied on expressing an acyl-ACP thioesterase to terminate FAS at the desired chain length. Howard and coworkers used a similar approach with heterologous thioesterases and branched FFAs in *E. coli* generating branched alkanes [17]. Eschewing heterologous thioesterases, Liu *et al.* engineered ACPs that prefer particular fatty acid chain lengths that can generate predictable alterations to the hydrocarbon cellular output [18]. While production of biofuels from fatty-acyl-CoA has enhanced control over the length of hydrocarbon chains produced, this pathway requires the additional processing step of CoA ligation compared to production from FFAs.

Recently, a more direct method to generate fatty aldehydes has used a carboxylic acid reductase (CAR) from *Mycobacterium marinum* to directly convert FFAs to fatty aldehydes (Figure 1b) [18]. Akhtar *et al.* demonstrated that this CAR can accept a broad range of substrate chain lengths. Furthermore, the CAR route is a more thermodynamically favored pathway than the cyanobacterial AAR route (−35.9 kJ/mol compared to −3.9 kJ/mol), and has much better *in vitro* kinetics than both the AAR and ACR1 pathways [19]. In a subsequent study, Kallio *et al.* used a thioesterase specific for butyryl-ACP to tune the FFA pool, which the CAR converted to generate short chain alkanes, producing 32 mg/L of propane in a shake flask fermentation [20••].

While biofuel production of alkanes is dependent on titers of FFAs, biofuel yields are much lower than the yields of FFAs due to factors including intermediate and