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Metabolic engineering: Use of system-level approaches and application to fuel production in *Escherichia coli*

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Abstract

Metabolic engineering was formally defined more than two decades ago (Bailey, 1991) and it is now an established discipline. Metabolic engineering is generally defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology (Bailey, 1991; Stephanopoulos et al. 1998). Therefore, the analysis and engineering/synthesis of metabolic pathways is of central importance to metabolic engineering. The analytical part uses a number of experimental and modeling techniques for the systematic study of cellular responses (in terms of RNA, protein and metabolite levels, metabolic fluxes, etc.) to genetic and environmental perturbations. This facilitates a rational design of metabolic modifications, which are implemented using recombinant DNA technology. Both, the analysis and the synthesis of metabolic pathways will be covered in this review. Recent efforts on the engineering of fermentative and biosynthetic pathways for biofuel production in *Escherichia coli*, as well as those enabling the utilization of novel feedstocks, will be highlighted.

Keywords: biofuels, *Escherichia coli*, metabolic engineering, systems biology.

SYSTEM-LEVEL ANALYTICAL AND SYNTHETIC TOOLS IN THE METABOLIC ENGINEERING TOOLBOX

Given the emphasis that metabolic engineering places on a system-level approach to analyze and engineer metabolism, several genome-wide techniques have been extensively used to analyze the metabolism of *Escherichia coli* and support engineering efforts (Figure 1). Below some of these analytical and synthetic tools in the metabolic engineering toolbox are presented.

Metabolic engineering undoubtedly benefits from traditional techniques that enable engineering and de novo synthesis of promoters, ribosome binding sites and entire coding regions, thus allowing modulation of both expression level and activity of target proteins/enzymes. However, new tools that enable genome engineering and system-wide identification of genes that confer target traits are very useful (Boyle and Gill, 2012). These emerging techniques include multiscale analysis of library enrichment (Scales) (Lynch et al. 2007), trackable multiplex engineering (TRMR) (Warner et al. 2010), coexisting/coexpressing genomic libraries (CoGeL) (Nicolau et al. 2011), genome-scale analysis of library sorting (GALibSo) (Stadlmayr et al. 2010), multiplex automated genome engineering (MAGE) (Wang et al. 2009), conjugative assembly genome engineering (CAGE) (Isaacs et al. 2011), and global transcription machinery engineering (gTME) (Alper and Stephanopoulos, 2007). Scales are libraries that provide quantification of the effects that the expression of a specific gene or gene
Metabolic fluxes are probably the best metric to capture the metabolic state of a cell. Flux quantification can be performed using a number of approaches, which can be broadly classified into in silico- and in vivo-based metabolic flux analysis (isMFA and ivMFA, respectively). One of the most representative examples of isMFA is flux balance analysis (Raman and Chandra, 2009), a technique used to construct genome-scale metabolic models and assess the metabolic capabilities of the organism of interest. For example, Feist et al. (2010) reported the use of isMFA to evaluate the production potential for growth-coupled products of E. coli. A recent development in this area is the introduction of the OptForce procedure (Ranganathan et al. 2010), which identifies all possible engineering interventions by classifying reactions in the metabolic model depending upon whether their flux values must increase, decrease or become equal to zero to meet a pre-specified overproduction target. The OptForce framework was validated by indentifying non-intuitive engineering strategies for succinate production in E. coli. An updated genome-scale network reconstruction of E. coli was also recently reported, demonstrating how it can be used to discover non-annotated genes in all strains sequenced to date (Orth et al. 2011).

One of the most widely used versions of ivMFA is a technique known as metabolite balancing, in which intracellular fluxes are estimated based on a stoichiometric model and extracellular measurements (Stephanopoulos et al. 1998; Klapa et al. 1999). This technique is based on mass balances around intracellular metabolites (which are considered in pseudo-steady-state) with the measurements of extracellular fluxes acting as constraints for flux calculation.

In combination with isMFA, metabolite balancing can provide significant insights into microbial metabolism (Murarka et al. 2010a). However, given the small number of extracellular measurements, when compared to the number of intracellular fluxes, this technique relies on the incorporation of redox (NADH/NADPH) and/or ATP balances as part of the stoichiometric model. This, in turn, can lead to incorrect flux estimation due to the incomplete knowledge about pathways involving NADH/NADPH and ATP. Another drawback of metabolite balancing is its inability to account for parallel metabolic pathways, metabolic cycles, and reversible or bidirectional reactions (Wiechert et al. 2001).

The use of isotopically labeled substrates (usually with 13C) provides additional constraints to the stoichiometric equations used in metabolite balancing, avoiding assumptions about redox and energy balances and potentially accounting for parallel pathways, cycles, and reversibility (Klapa et al. 1999).

This approach is based on feeding an organism with a mixture of a specifically 13C-labeled and a naturally abundant version of the same substrate and measuring the 13C enrichments or isotopomer distributions in the carbon atoms of different metabolites. The most common metabolites used for this purpose are proteinogenic amino acids because they are abundant, stable, and their labeling pattern reflects that of precursors metabolites generated in central metabolism. In one approach called metabolic flux ratio (MetaFoR) analysis relative local fluxes around a node are obtained from the abundances of intact carbon fragments in metabolites (calculated from the 2-D [13C, 1H] NMR data) (Fischer and Sauer, 2003; Fischer and Sauer, 2005). Comprehensive 13C-based metabolic flux analysis (13C-MFA), on the other hand, is achieved through a modeling approach that requires information on the metabolic network, labeling patterns of amino acids, and extracellular fluxes. This information is combined in an error function that accounts for the average difference between measured and simulated labeling patterns. An optimization procedure is then followed to solve for intracellular fluxes that minimize this error function (Klapa et al. 1999; Tang et al. 2009). 13C-MFA has
been extensively used to characterize the fermentative metabolism of *E. coli* (Murarka et al. 2010b; Choudhary et al. 2011).

An excellent example of the value of different MFA approaches to the design and implementation of ME strategies was recently reported on an integrated computational and experimental study for overproducing fatty acids in *E. coli* (Ranganathan et al. 2012). The OptForce procedure was used for suggesting and prioritizing genetic manipulations that could support overproduction of fatty acids of different chain lengths (C6-C16) starting with wild-type *E. coli*. When interventions suggested by OptForce were implemented, the engineered strain produced 1.70 g/L of C(14-16) fatty acids in minimal medium at a yield of 0.14 g fatty acid/g glucose.

![Synthesis and Analysis of Metabolism](image)

**Fig. 1** Interplay between *synthesis* and *analysis* of metabolism, the key components of the metabolic engineering cycle. Representative approaches for synthesis and analysis are shown.

### Metabolic control analysis

The identification of metabolic bottlenecks and rate-limiting steps has always been of paramount importance for metabolic engineering and Metabolic Control Analysis (MCA) has for long provided a framework to conducting such analyses. MCA can be used to elucidate the control structure of a metabolic network (Fell and Sauro, 1985). This is achieved through the calculation of flux and concentration control coefficients, the former being of special importance because they indicate how a relative change in the activity of an enzyme affects the flux of a pathway (Stephanopoulos et al. 1998). Such an analysis can identify reactions that control the carbon flux through the pathway and hence genes/proteins that need to be manipulated to increase the flux towards the desired product. The MCA approach was recently used to conduct a quantitative analysis of the fermentative metabolism of glycerol in *E. coli*, gain a better understanding of glycerol fermentation and identify key targets for genetic manipulation that could enhance product synthesis (Cintolesi et al. 2012). The calculated flux control coefficients predicted that the glycolytic flux during glycerol fermentation is almost exclusively controlled by the enzymes glycerol dehydrogenase (encoded by *gldA*) and dihydroxyacetone kinase (encoded by *dhaKLM*). In agreement with the MCA findings, overexpression of *gldA* and *dhaKLM* led to significant increase in glycerol utilization and ethanol synthesis fluxes.

An MCA-based method, called ORACLE (optimization and risk analysis of complex living entities), has also been proposed to calculate control coefficients without the need for a kinetic model (Miskovic and Hatzimanikatis, 2010).

### “Omics” analyses

Advances of the genomic revolution have opened new opportunities for the challenging task of assigning biological function to each gene and elucidating the metabolic and regulatory networks that operate in a biological system. Functional genomics has then emerged as a new discipline that relates
to “the development and application of high-throughput global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics” (Hieter and Boguski, 1997). The field of functional genomics has grown exponentially in the last decades, embracing areas such as transcriptomics (mRNAs), proteomics (proteins), metabolomics (metabolites), and fluxomics (fluxes), among others. This systems-level approach to understand complex metabolic and regulatory networks enables the design of more efficient microorganisms for the production of valuable molecules, including biofuels.

Significant progress in the development of functional genomic tools in recent years has powered the elucidation of complex phenotypes and the engineering of new ones for the development of promising industrial strains (Stephanopoulos et al. 2004; Zhang et al. 2006). The use of transcriptional profiling tools, such as DNA microarrays, allows the study of gene expression by identifying differentially expressed genes under different experimental conditions or resulting from certain genetic perturbations. However, other functional genomics tools are required to complement transcriptomic studies. Proteomics is of special importance because most of the cellular activities are mediated by proteins, and is the primary tool used to describe cellular interactions beyond mRNA level (Graham et al. 2007). The large-scale quantification and identification of proteins can be achieved by one- or two-dimensional gel electrophoresis, followed by software aided quantification tools and mass spectrometry techniques (Watt et al. 2003), or by gel-free techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and protein isotope labeling (Gevaert et al. 2007). Comparative proteomic analyses detect differentially expressed proteins by variations in the intensities of labeled protein spots in the gel, under different genetic or environmental conditions. Similar to proteins, metabolites are also context-dependent entities whose concentration is directly related to the physiological state of the cell (Raamsdonk et al. 2001). Metabolomics makes use of high-throughput analyses like Nuclear Magnetic Resonance (NMR), and Gas or Liquid Chromatography coupled to Mass Spectrometry (GC-MS, and LC-MS) for the quantification of intra- and extra-cellular small-molecular-weight metabolites (Buchholz et al. 2002). Metabolic studies also serve as a screening tool to investigate the unknown effect of a specific genetic modification on the phenotype of the organism, and as a means to describe metabolism kinetics by performing pulse experiments (Buchholz et al. 2002).

ENGINEERING OF FERMENTATIVE AND NON-FERMENTATIVE PATHWAYS FOR FUEL PRODUCTION IN E. COLI

The engineering of pathways that convert key intermediates into the fuel of interest is typically the first step taken to ensure product synthesis at maximum yield and rate. Both fermentative and biosynthetic pathways have been engineered in E. coli for the production of a variety of alcohols, fatty acids and a host of novel fuels derived from the fatty acid, polyketide, and isoprenoid biosynthesis pathways, as well as engineered ketoacid and reversal of the β-oxidation pathways, among others.

Engineering of fermentative pathways

One of the first applications of metabolic engineering was reported about 25 years ago, when the laboratory of Lonnie Ingram engineered E. coli to produce ethanol as the primary product of glucose fermentation (Ingram et al. 1987). Under fermentative conditions, growth of wild-type E. coli on simple sugars, such as glucose and xylose, results in the production of acids (lactic, formic, succinic and acetic) and small amounts of ethanol (Sawers and Clark, 2004). To engineer a homoethanologenic pathway, Ingram and co-workers (Ingram et al. 1987) transferred the ethanol synthesis pathway from Zymomonas mobilis (a two step pathway in which pyruvate decarboxylase (PDC) converts pyruvate directly to acetaldehyde and CO₂ and alcohol dehydrogenase (ADH) then converts acetaldehyde into ethanol: Figure 2) to a strain of E. coli containing a mutation in the frd operon (encoding the enzyme fumarate reductase, which is responsible for the conversion of fumarate to succinate). These two sets of genetic manipulations led to a complete shift of the fermentative metabolism of E. coli from mixed acid to homoethanologenic fermentation (Ingram et al. 1987; Ohta et al. 1991) and paved the way for the metabolic engineering of E. coli to efficiently synthesize ethanol and a host of fuel molecules.

While ethanol was the first biofuel to reach commercial production, its incompatibility with current fuel infrastructure poses a significant challenge for its widespread adoption. Higher-chain alcohols, on the other hand, represent a potential alternative and hence their biological production has been explored.
Some of these alcohols are natively produced by a number of Clostridium strains (Chen and Hiu, 1986; Jones and Woods, 1986). However, given the lack of genetic capabilities available for the optimization of these organisms, the engineering of E. coli was recently investigated with promising results. For example, E. coli was engineered to produce isopropanol at levels higher than any seen in naturally producing organisms (4.9 g/L) by the expression of the acetone production pathway of Clostridium acetobutylicum along with a secondary alcohol dehydrogenase to convert acetone into isopropanol (Figure 2) (Hanai et al. 2007).

The synthesis of 1-butanol via engineered fermentative pathways has also been reported in E. coli. Initial work focused on the transfer of the 1-butanol synthesis portion of the Clostridial Acetone-Butanol-Ethanol (ABE) pathway into strains of E. coli devoid of native fermentative pathways (Atsumi et al. 2008a; Inui et al. 2008) (Figure 2). Expression of native E. coli AtoB (atoB), along with all the necessary genes for butanol production from acetyl-CoA (thl, hbd, crt, bcd, etfAB, and adhE2), and disruption of native pathways competing with the butanol pathway for acetyl-CoA and NADH (deletion of ldhA, adhE, trdBC, pta, and fur) led to a maximum butanol titer of 550 mg/L (Atsumi et al. 2008a).

Similar levels of 1-butanol production were achieved upon expression of the same pathway from Clostridium tertioventriculorum (Cb) and Clostridium beijerinckii (Cb); Treponema denticola (Td); Zymomonas mobilis (Zm); and Saccharomyces cerevisiae (Ca), along with expression of formate dehydrogenase (fdh1), which increases the availability of reducing equivalents, and glyceraldehyde 3-phosphate (gapA) from E. coli to increase glycolytic flux (Nielsen et al. 2009). Another study reported the production of 1.2 g/L of 1-butanol after 60 hrs upon expression of the entire heterologous pathway from a single plasmid (Inui et al. 2008).

Fig. 2 Pathways engineered in E. coli for the fermentative production of ethanol, isopropanol, and 1-butanol (shown in grey shaded boxes). Broken lines represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme. Non-E. coli genes are noted in parenthesis using the following abbreviations: Clostridium acetobutylicum (Ca), Clostridium beijerinckii (Cb); Treponema denticola (Td); Zymomonas mobilis (Zm); accep-ldpA, pyruvate dehydrogenase multienzyme complex; ackA, acetate kinase; adc, acetate dehydrogenase (Ca); adh, secondary alcohol dehydrogenase (Cb); adhB, alcohol dehydrogenase (Zm); adhE, alcohol dehydrogenase; adhE2, secondary alcohol dehydrogenase (Ca); atoA, atoD, acetyl-CoA:acetoacetyl-CoA transferase; atoB, acetyl-CoA acetyltransferase; bcd, butyryl-CoA dehydrogenase (Ca); ctfAB, coenzyme A acyltransferase (Ca); ctfAB, electron transfer flavoprotein (Ca); hbd, β-hydroxybutyryl-CoA dehydrogenase (Ca); pyr, pyruvate dehydrogenase (Ca); pflB, pyruvate formate lyase; pta, phosphatase (Ca); thl, acetyl-CoA acetyltransferase (Ca); trdBC, acetate dehydrogenase (Ca); and, trasnenoyl-CoA reductase (Td). Abbreviations: 2[H] = NAD(P)H = FADH; DHAP, dihydroxyacetone-phosphate; Gly-3-P, glyceraldehyde-3-phosphate; PYR, pyruvate.
While the success of the above-mentioned efforts was limited by the low activity of the foreign pathway in *E. coli*, identification of key limiting steps and use of genes/enzymes not involved in the native 1-butanol synthesis pathway has recently enabled the efficient synthesis of 1-butanol (Bond-Watts et al. 2011; Shen et al. 2011). Of particular relevance was the use of a transenoyl-CoA reductase from *Treponema denticola* to replace the clostridial butyryl-CoA dehydrogenase in the conversion of crotonyl-CoA to butyryl-CoA (Bond-Watts et al. 2011; Shen et al. 2011). Process-based improvements, such as the removal of 1-butanol from the fermentation broth, led to the synthesis of 30 g/L of 1-butanol (Shen et al. 2011).

**Engineering of non-fermentative pathways**

While fermentative pathways can be exploited for the synthesis of valuable fuel candidates, the use of non-fermentative pathways represents an invaluable opportunity to generate “designer” fuels with properties that make them compatible with current infrastructure for fuel transportation, storage and use. Such pathways include the fatty acid, polyketide, and isoprenoid biosynthesis pathways, as well as engineered keto-acid and reversal of the β-oxidation pathways (Figure 3).

**Fuels derived from the fatty acid biosynthesis pathway.** The high-energy content and wide array of chain length and degree of saturation of fatty acids could support the synthesis of molecules similar in structure and composition to those currently found in petroleum-based fuels. The activated form of fatty acids, thiosters with coenzyme A (fatty acyl-CoAs) or acyl carrier protein (fatty acyl-ACPs), are key intermediates in the biosynthesis of fatty acid derived fuels (Figure 3). A number of recent reports have shown the potential of engineering the native fatty acid biosynthesis pathway in *E. coli* to produce fatty acids (Lennen et al. 2010; Steen et al. 2010; Zhang et al. 2011), fatty esters (Steen et al. 2010), long-chain alcohols (Steen et al. 2010), waxes (Steen et al. 2010), hydrocarbons (Lennen et al. 2010; Schirmer et al. 2010), methyl ketones (Park et al. 2012), and biodiesel (Steen et al. 2010).

In an early study, *E. coli* was engineered to produce fatty esters (biodiesel), fatty alcohols, and waxes directly from simple sugars or hemicellulosate (Steen et al. 2010). Production of free fatty acids and acyl-CoAs through the native fatty acid biosynthesis pathway was achieved by eliminating β-oxidation, and by overexpressing thioesterases and acyl-CoA ligases. The synthesis of biodiesel, alcohols and wax esters was achieved by overexpression of non-native pathways. For example, alcohols were produced from fatty acyl-CoAs by overexpressing fatty acyl-CoA reductases, esters by expressing an acyltransferase in conjunction with an alcohol-forming pathway, and biodiesel by introducing the ethanol pathway shown in Figure 2.

The synthesis of alkanes, on the other hand, was facilitated by the discovery of an alkane biosynthesis pathway from cyanobacteria that consists of an acyl-acyl carrier protein reductase that reduces acyl-ACPs to fatty aldehydes, which are then decarbonylated to odd-chain alkanes and alkenes by an aldehyde decarboxylase (Schirmer et al. 2010). Although the mechanism employed by the decarboxylase is still under investigation (Das et al. 2011; Li et al. 2011), the expression of the two-step pathway in *E. coli* produced C\textsubscript{13}-C\textsubscript{17} mixtures of alkanes and alkenes (Schirmer et al. 2010).

**Fuels derived from the isoprenoid biosynthesis pathway.** While isoprenoids are naturally synthesized by plants, animals, and bacteria, and used for their pharmaceutical and nutritional value (Rude and Schirmer, 2009), only recently has there been an interest in producing isoprenoid-derived biofuels. Two different pathways have been utilized to overproduce isoprenoids from the precursor metabolites isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Figure 3). The mevalonate pathway generates the aforementioned precursors using acetyl-CoA, while the methylenetrithiol phosphate pathway produces them from glyceraldehyde-3-phosphate (G3P) and pyruvate (Dewick, 2002). Diterpene precursors geranyl pyrophosphate (GPP), farnesy1 phosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) are then generated from IPP and DMAPP through the action of prenyltransferases (Rude and Schirmer, 2009). Isoprenoid alcohols and olefins, which are fuel molecules, can be synthesized from IPP, GPP, FPP, or GGPP through the action of phosphatases, pyrophosphatases, and terpene synthases (Christianson, 2008; Rude and Schirmer, 2009). For example, isopentenol was produced in an *E. coli* strain engineered to overproduce IPP and DMAPP via the mevalonate pathway, in combination with the isopentenol biosynthetic pathway from *Bacillus subtilis* (*yhfR* and *nufF*) (Martin et al. 2003; Withers et al. 2007).
More recently, *E. coli* has been engineered for the production of farnesol (Wang et al. 2010), farnesene (Renninger and McPhee, 2008), pinene (Reiling et al. 2004), limonene (Carter et al. 2003), and bisabolene (Peralta-Yahya et al. 2011). In the case of farnesol, heterologous mevalonate pathways and FPP synthase were over expressed in combination with endogenous phosphatases for FPP hydrolysis (Wang et al. 2010). Bisabolene, on the other hand, was produced at levels approaching 1 g/L by expressing in *E. coli* a codon-optimized bisabolene synthase from *Abies grandis* in combination with an optimized heterologous mevalonate pathway (Peralta-Yahya et al. 2011).

**Fuels derived from amino acid/ketoacid biosynthesis pathways.** The native amino acid biosynthetic pathways in *E. coli* can be harnessed to generate 2-ketoacids, which serve as precursors for higher chain alcohol production through a two step synthetic pathway that mimics the Ehrlich degradation pathway: *i.e.* conversion of 2-ketoacids to an alcohol, with an aldehyde as an intermediate, through the action of a 2-ketoacid decarboxylase (KDC) and an alcohol dehydrogenase (ADH) (Figure 3) (Atsumi and Liao, 2008b). A 2-ketoacid decarboxylase from *Lactococcus lactis* and an alcohol dehydrogenase Adh2 from *S. cerevisiae* exhibited broad substrate activity toward a number of 2-ketoacids and enabled the production of 1-propanol, isobutanol, 1-butanol, 3-methyl-1-butanol, and 2-phenylethanol in an engineered *E. coli* (Atsumi et al. 2008b) (Figure 3). The yield and productivity of these alcohols was improved by increasing the production of the 2-ketoacid intermediates using different strategies. For example, expression of *als* (*B. subtilis*) and *ilvCD* (*E. coli*) to generate 2-ketoisovalerate, combined with the expression of the aforementioned KDC and ADH, led to an engineered strain able to produce ~22 g/L of isobutanol after 110 hrs at a yield of 86% of the theoretical maximum (Atsumi et al. 2008b). Similar strategies were used to demonstrate the efficient synthesis of 1-propanol and 1-butanol, observing the production of 3.5 g/L of 1-propanol and ~500 mg/L of 1-butanol after 92 hrs (Atsumi and Liao, 2008a; Shen and Liao, 2008). These natural amino acid pathways were also utilized to produce the 5-carbon alcohols, 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) (Figure 3) (Cann and Liao 2008; Connor and Liao 2008; Connor and Liao, 2009).

The above strategy, based on the use of the amino acid biosynthetic pathways, has also been exploited to produce non-natural 5 to 8 carbon higher alcohols derived from isoleucine biosynthesis. To this end, the product chain length of LeuA and substrate chain length of 2-ketoacid decarboxylase (*KIVD*) was expanded to enable the synthesis of non-natural ketoacids, which were then converted to higher chain alcohols such as 1-pentanol, 1-hexanol, (S)-3-methyl-1-pentanol, 4-methyl-1-pentanol, (S)-4-methyl-1-hexanol, and (S)-5-methyl-heptanol (Zhang et al. 2008).

**Fuels derived from an engineered β-oxidation pathway.** A variation of the β-oxidation cycle has been engineered to work in the reverse biosynthetic direction (as opposed to its natural catabolic direction) in order to produce fuels and chemicals from different carbon sources (Figure 3) (Dellomonaco et al. 2011; Clomburg et al. 2012). In the earlier report (Dellomonaco et al. 2011), a system-level approach was used in which several points for regulation of the β-oxidation cycle, namely *lad*, *ato*, *cip*, and *arcA*, were engineered. These modifications, in combination with the overexpression of endogenous dehydrogenases and thioesterases, enabled the synthesis of *n*-alcohols, FAs, and carboxylic 3-hydroxy-, 3-keto-, and trans-Δ3-carboxylic acids (Dellomonaco et al. 2011). In addition, the deletion of the competing pathways for ethanol (adhE), acetate (pta), and succinate (frdA) helped minimizing the formation of by-products, while the expression of selected termination enzymes enabled the production of a variety of β-oxidation cycle intermediates. The introduction of the engineered reversal of the β-oxidation cycle opened many opportunities for engineering *E. coli* metabolism for the production of fuels and chemicals in an ATP-independent and efficient manner, but given the implications of modifying major regulators in the cell it could be difficult to completely elucidate and understand the responsible genes/proteins. To address these issues, a more recent study presented *in vitro* kinetic characterization of the functional units in the pathway and used this information to guide a synthetic biology approach to assemble the pre-defined components of the reversed β-oxidation pathway *in vivo* conditions and to enable production of a variety of carboxylic acids with the combination of different thioesterases (Clomburg et al. 2012). A functional equivalent of the β-oxidation reversal that does not rely on β-oxidation enzymes, has also been reported (Dekishima et al. 2011; Machado et al. 2012).
Fig. 3 Non-fermentative routes for the production of alcohols via the ketoacid pathway (dotted line box with grey shade), fatty acid- and β-oxidation reversal-derived fuels (solid line box with grey shade) in E. coli. Broken lines represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme. Non-E. coli genes are noted in parenthesis using the following abbreviations: Acinetobacter calcoaceticus (Ac); Arabidopsis thaliana (At); Bacillus subtilis (Bs); Clostridium acetobutylicum (Ca); Corynebacterium glutamicum (Cg); Euglena gracilis (Eg); Lactococcus lactis (Ll); Methanococcus jannaschii (Mj); Saccharomyces cerevisiae (Sc); Stenotrophomonas maltophilia, (Sm). accABCd, acetyl-CoA carboxylase; acr1, acyl-CoA reductase (Ac); ADH2, alcohol dehydrogenase (Sc); adhE2, alcohol dehydrogenase (Ca); atfA, acyltransferase (Ab); atfB, yfegF/fadA, thiolase; betaA/euitG/3Ya, alcohol dehydrogenase (At); cimA, citramalate synthase (Mj); ddx, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; dxx, 1-deoxy-D-xylulose 5-phosphate synthase; ERG12, mevalonate kinase (Sc); ERG8, phosphomevalonate kinase (Sc); fabA, hydroxydecanoyl-ACP dehydrase; fabB, ketoacyl-ACP synthase I; fabD, malonyl-CoA-ACP transacylase; fabF, ketoacyl-ACP synthase II; fabG, ketoacyl-ACP reductase; fabL, hydroxyacyl-ACP dehydratase; fabD, acyl-CoA synthetase; fabE, hydroxyacyl-CoA dehydrogenase & enoyl-CoA hydratase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase (Sc); id1, isopenenylpyrophosphate isomerase; isPD, 4-diphosphocytidyl-2-methylenerythritol synthase; ispE, 4-diphosphocytidyl-2-methylenerythritol kinase; ispF, 2-methylenerythritol 2,4-cyclophosphate synthase; ispG, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; ispH, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; kivd, ketoisovalerate dehydratase; kivL, malonate pyrophosphate decarboxylase (Sc); nudF, prenyl phosphatase (Bs); ter, trasnonenoyl-CoA reductase (Eg); thmGR, truncated 3-hydroxy-3-methylglutaryl-CoA reductase (Sc); yidO, enoyl-CoA reductase; † represents a number of endogenous acyl-CoA and acyl-ACP thioesterases; ‡ represents numerous reactions leading to the formation of a wide range of isoprenoids. Abbreviations: Ac-CoA, acetyl-CoA; ACP, acyl carrier protein; CoA, coenzyme A; DMAPP, dimethylallyl pyrophosphate; FAEE, fatty acid ethyl ester; Gly-3-P, glyceraldehyde-3-phosphate; IPP, isopentenyl pyrophosphate; PEP, phosphoenolpyruvate.

The β-oxidation pathway has also been exploited for the production of methyl ketones in E. coli (Goh et al. 2012). The authors used a strategy in which synthesis of methyl-ketones is achieved by overexpression of acyl-CoA oxidase to generate β-ketoacyl-CoA, which is then hydrolyzed by an endogenous thioesterase (FadM) and the resulting ketoacid decarboxylated to C11-C15 methyl ketones at a titer of 380 mg/L and 58% of the maximum theoretical yield.

ENGINEERED PATHWAYS THAT ENABLE THE USE OF ALTERNATIVE FEEDSTOCKS FOR FUEL PRODUCTION IN E. COLI

The engineering efforts discussed above mainly focused on engineering pathways that convert metabolites in central metabolic pathways to the target fuel molecule. Another important consideration...
is the use of more efficient substrates that can lead to higher product yields. Two abundant substrates, glycerol and fatty acids, can be considered in this category.

The reduced nature of carbon atoms in glycerol and fatty acids provides significant advantages for the production of fuels, such as alcohols, alkanes, and others, when compared to their production from sugars (Clomburg and González, 2013). For example, fermentative metabolism of glycerol leads to the synthesis of ethanol as the preferable product due to the fact that ethanol synthesis from glycerol represents an NADH balanced pathway that produces ATP via substrate level phosphorylation. Metabolic engineering strategies have been implemented to minimize by-product formation through the disruption of genes encoding fumarate reductase (frdA) and phosphotransacetylase (pta), key enzymes in the synthesis of succinate and acetate, respectively (Yazdani and González, 2008). The resulting strain co-produced ethanol and hydrogen in nearly equimolar amounts approaching the maximum theoretical yield of 1 mol each of ethanol and hydrogen per mol of glycerol fermented. Using a similar strategy, with the additional disruption of the enzyme responsible for the oxidation of formate into H₂ and CO₂, the efficient co-production of ethanol and formate was achieved (Yazdani and González, 2008). While promising, strains reported in that study (Yazdani and González, 2008) required rich supplementation in the form of tryptone to be able to produce ethanol and co-products formate or hydrogen. Microaerobic conditions were then used as a means to eliminate the need for rich nutrients while still taking advantage of the higher degree of reduction of glycerol for product synthesis (Durnin et al. 2009). In another study, elementary mode analysis was used to design an E. coli strain for the conversion of glycerol into ethanol (Trinh and Sriend, 2009). A coupling between cell growth and ethanol production enabled metabolic evolution of the designed strain to convert 40 g/L glycerol to ethanol at 90% of the theoretical yield.

E. coli has also been engineered for the production of ethanol, isopropanol, and 1-butanol from fatty acid feedstocks, resulting in product yields much higher than the theoretical maximum obtained from sugars (Dellomonaco et al. 2010). In order to produce ethanol under the respirato-fermentative conditions required for fatty acid utilization, an aerotolerant mutant of alcohol dehydrogenase (encoded by adhE) was constructed. Overexpression of AdhE* from an oxygen-independent promoter in a strain engineered for efficient and complete degradation of fatty acids resulted in the production of ethanol with yields two-times greater than the theoretical maximum value than can be achieved with the use of lignocellulosic sugars (Dellomonaco et al. 2010). In the same study, E. coli was also engineered to produce isopropanol and 1-butanol from fatty acids through the expression of the fermentative pathways for the synthesis of these molecules from acetyl-CoA shown in Figure 2 and described in previous sections.

CONCLUDING REMARKS

As the above sections show, metabolic engineering has become an established discipline and enabled the development of microbial systems for fuel production from a number of renewable feedstocks. Advancements in the field of synthetic biology are key to current and future success. In addition, the use of system biology tools such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics will facilitate the design, characterization and integration of new metabolic pathways for biofuel production. The development of new strategies and tools from these fields will support further increases in yield, titer, and productivity of existing and new biofuel molecules from various feedstocks. This, in turn, represents a promising avenue for the viable industrial production of renewable fuels.

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