Genetic Engineering of *Escherichia coli* for Biofuel Production

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Abstract

In order to mitigate climate change without adversely affecting global energy supply, there is growing interest in the possibility of producing transportation fuels from renewable sources via microbial fermentation. Central to this challenge is the design of biocatalysts that can efficiently convert cheap lignocellulosic raw materials into liquid fuels. Owing to the wealth of genetic and metabolic knowledge associated with *Escherichia coli*, this bacterium is the most convenient starting point for engineering microbial catalysts for biofuel production. Here, we review the range of liquid fuels that can be produced in *E. coli* and discuss the underlying biochemistry that enables these metabolic products. The fundamental and technological challenges encountered in the development of efficient fermentation processes for biofuel production are highlighted. The example of biodiesel is a particularly illustrative case study and is therefore discussed in detail.

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Octane number: a measure of the resistance of a motor fuel to knocking. Octane numbers are based on a scale on which isooctane is 100 (minimal knock) and heptane is 0 (bad knock)

Energy density: the amount of energy per unit volume of a material

Cetane number: a measure of the ignition delay of a fuel, i.e. the time between the start of injection of the fuel and its ignition

INTRODUCTION

The twentieth century witnessed an explosive growth in the use of petroleum, the demand for which is anticipated to continue increasing in the foreseeable future. Diminishing petroleum reserves, unequal distribution of fossil fuel throughout the world and, perhaps most importantly, the unsustainable net rates of greenhouse gas emission have prompted an interest in the development of transportation fuels from renewable sources (41, 76). By now, the overarching concept of carbon neutrality has gained sufficient political and social momentum so as to enable the recent entry of at least two types of biofuels into the marketplace—ethanol from sugarcane or corn, and biodiesel from soybean, rapeseed, or palm oil (32). Given that neither product can be scaled up to completely replace petroleum-derived transportation fuels, it is widely anticipated that biofuels of the future will likely be derived from agricultural waste or dedicated lignocellulosic crops (24).

Fundamentally, two steep challenges are encountered in the conversion of a low-value lignocellulosic feedstock into a transportation fuel. First, polymeric cellulose, hemicellulose, and lignin must be broken down into simpler compounds such as sugars or organic acids. Second, these monomers (or oligomers) must be deoxygenated into liquid fuel. For such fuels to economically compete with fossil fuels, both processes must operate with maximum possible atom economy and volumetric productivity. (Bio)catalysis is the likely answer to this challenge. For an introduction to chemical processes that convert biomass into fuel, the reader is directed elsewhere (68). This review focuses on the design of microbial catalysts for the same purpose.

Parenthetically, it should be noted that there is growing interest in the use of phototrophic organisms such as cyanobacteria or algae to directly fix carbon dioxide into liquid fuel (17). For example, *Synechococcus elongatus* PCC7942 has been engineered to produce isobutyraldehyde and isobutanol (6). Whereas the potential impact of such approaches cannot be overestimated, they represent longer-term solutions to the problem of unsustainable greenhouse gas emission (17, 28). By contrast, at least one product of heterotrophic fermentation (ethanol) is already sold as a liquid transportation fuel (see sidebar, Types of Liquid Transportation Fuels) and therefore serves as a technical and economic benchmark for next-generation, fermentation-derived fuels that are being evaluated or scaled up. As genetic engineering plays a critical role in all such efforts, this area represents an interesting new opportunity at the interface between genetics, biochemistry, and bioprocess engineering.

Central to the genetic engineering of a biocatalyst for fuel production is the choice of a microbial host. In this review, we focus on *Escherichia coli* because of its plethora of sophisticated genetic tools as well as its recent track record in the biotechnology industry. For example, *E. coli* is used today to produce diverse high-volume chemicals including polyhydroxybutyrate (a biodegradable plastic) (75), 1,3-propanediol (carpet fiber monomer) (58), and amino acids (animal feed additives) (60).

TYPES OF LIQUID TRANSPORTATION FUELS

The ideal biofuel is a fermentation-derived molecule or blend that is structurally identical, or at least functionally equivalent, to existing petroleum-derived transportation fuels. This would enable compatibility with existing internal combustion engine designs as well as infrastructure for fuel transportation and storage. Three types of transportation fuels dominate the marketplace—gasoline, diesel, and jet fuel. Gasoline consists of C_4–C_{12} hydrocarbons, including linear, branched, and cyclic alkanes (40–60%), aromatics (20–40%), and antiknock additives (67). The most important properties of a particular gasoline blend are its octane number, energy density, and transportability. Diesel on the other hand consists of C_9–C_{23} hydrocarbons (average C_{16}), including linear, branched, and cyclic alkanes (75%), aromatics (25%), and antifreeze additives (44). Its most important properties are its cetane number, kinematic viscosity, oxidative stability, and cloud point (45). The major components of jet fuel are C_8–C_{16} hydrocarbons, with aromatics and antifreeze additives present up to a 25% (v/v) limit. Jet fuel has a lower freezing point than diesel to enable its use at high altitudes (46).

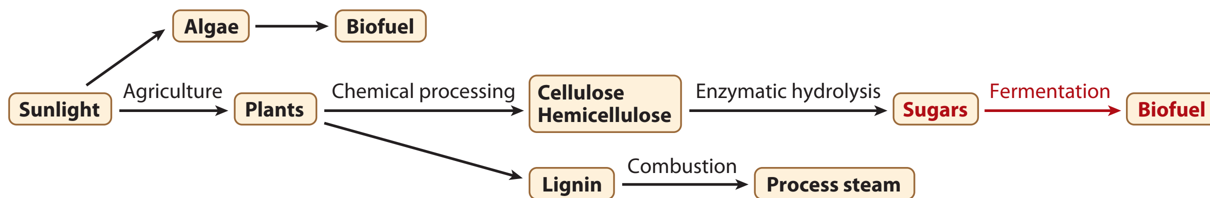

Figure 1

Production of biofuel from lignocellulosic biomass. The current review focuses on steps highlighted in red.

Thus, transportation fuels represent a logical challenge for expanding the scope of this prototypical bacterium as a catalyst for green chemistry. The processes by which solar energy is harnessed to produce biofuel are summarized in **Figure 1**. A variety of alternative hosts, such as *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, *Clostridia*, and *Trichoderma reesei* may also be considered, depending upon the nature of the raw material or the target fuel chemistry (3, 23). In this review, we only focus on the work that deals with the engineering of *E. coli* for making energy-rich, fuel-like molecules, and we try to bring some new inside information to the reader and discuss the problems that still exist and possible solutions.

TRANSFORMATION OF LIGNOCELLULOSIC FEEDSTOCKS INTO CARBON SOURCES

The social and economic benefits of making biofuel from lignocellulosic biomass instead of crops such as corn, sugarcane, or soybean are widely appreciated. To do so, however, cellulose and hemicellulose need to be hydrolyzed into fermentable sugars. First, the plant cell wall must be broken down via chemical and mechanical processing (33, 52). This includes pretreatment with steam, dilute acid, and ammonia to release the cellulose, hemicellulose, and lignin (31). In addition to the incremental costs of such pretreatment, sugar degradation and inhibitor release are two significant problems encountered in the utilization of conventional lignocellulosic feedstocks. Therefore, engineering easily cultured and cellulose-rich plants, such as switchgrass, represents an important goal for geneticists (14, 73). Alternatively, genetic modification of plants to alter lignin chemistry or enable growth on abandoned land may also favorably influence the economics of biofuel production (11).

Once cellulose and hemicellulose are released, they are typically depolymerized into glucose, xylose, and the other sugars by enzymatic hydrolysis. Regardless of the specific biofuel chemistry, the economics of this step heavily influences the ultimate cost of the biofuel. Consequently, both the discovery (61) and engineering (50) of superior cellulases and hemicellulases are important objectives, and are being enhanced by recent advances in genomics and related technologies (53, 82). Efficient transformation of cellulose, hemicellulose, and lignin into liquid fuel also warrants serious consideration of mixed fermentative processes in which specialized microbes secrete a battery of enzymes that convert these strong, insoluble polymers into fermentable substances, followed by conversion of the resulting compounds into fuel by one or more genetically engineered strains of *E. coli*.

THE SCOPE OF BIOFUEL CHEMISTRY IN *E. COLI*

An *E. coli* cell harbors more than four thousand genes, hundreds of metabolic pathways, and thousands of metabolites. **Figure 2** summarizes the range of energy-rich molecules that are either naturally present in *E. coli* or could readily be produced in this host by relatively straightforward genetic manipulation. (Very similar metabolic processes also operate in other

Oxidative stability: susceptibility of fuel to oxidation, which induces a rancid odor and flavor. The higher the level of unsaturation of a fuel, the lower its oxidative stability

Cloud point: a measure of the tendency of the fuel to plug filters or small orifices at low operating temperatures

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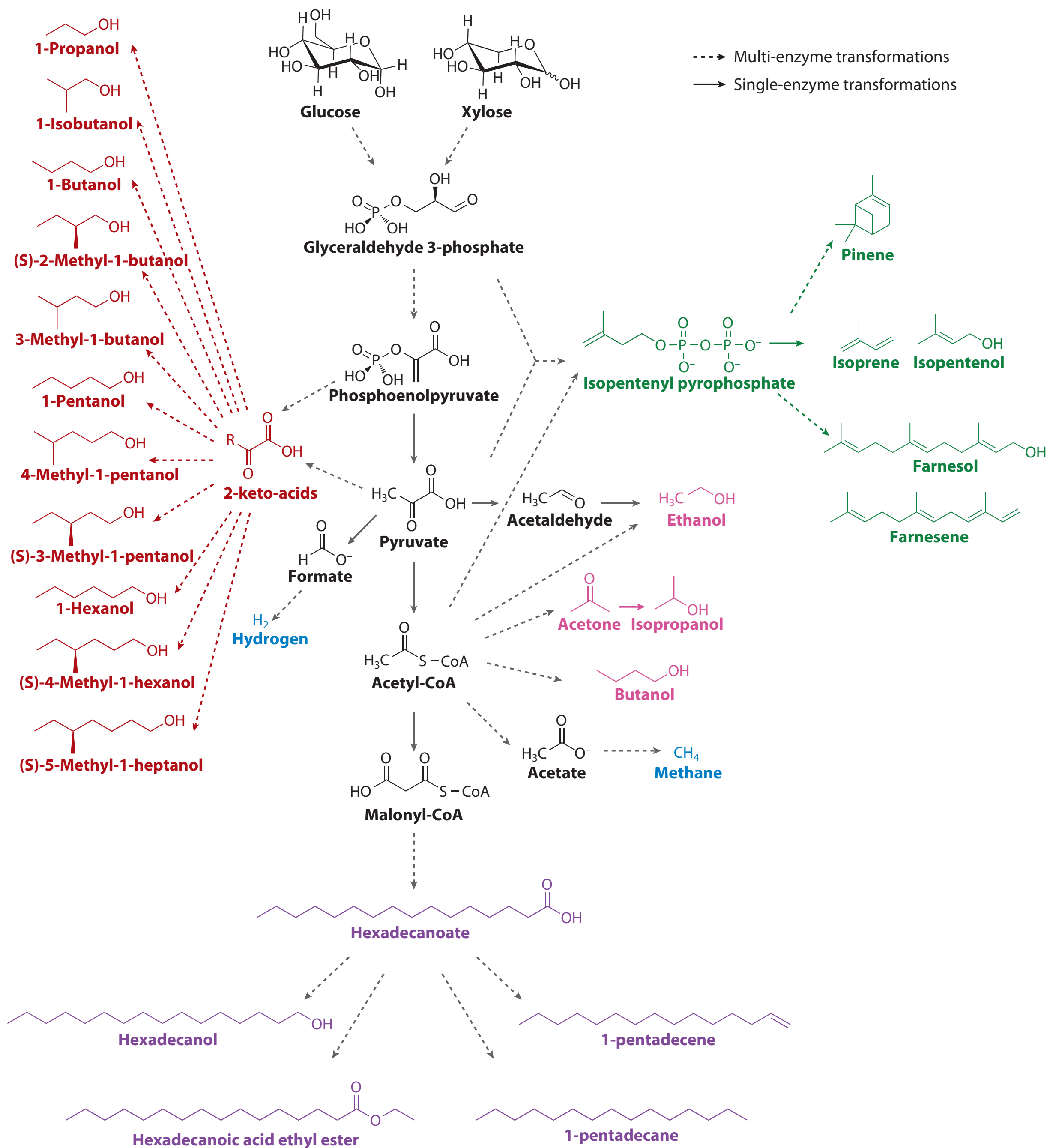

Figure 2

The scope of biofuel chemistry in *Escherichia coli*. Dashed arrows represent multi-enzyme transformations, whereas solid arrows indicate transformations catalyzed by a single enzyme. The different classes of fuel-like biomolecules discussed in this review are shown in different colors.

candidate hosts for biofuel production.) For example, *E. coli* can ferment glucose into a wide range of short-chain alcohols (4, 27, 34). Although ethanol is already blended with gasoline, other alcohols have inherent advantages as transportation fuels, as discussed below. *E. coli* can also produce highly deoxygenated or even fully deoxygenated hydrocarbons via fatty acid or isoprenoid biosynthetic pathways (24, 46, 66). Plant-derived fatty acid methyl esters (FAMEs) are already used as biodiesel in stand-alone or blended forms (32). Terpenes such as isoprene and farnesene also have similar properties to gasoline and jet fuel, respectively, and could potentially be commercialized as biofuels (46, 56, 64). Last but not least, *E. coli* could be engineered in principle to produce gaseous fuels such as hydrogen (90, 91) and methane (37). Importantly, all of the above candidate biofuels are derived from central carbon metabolism, which ordinarily converts one hexose molecule into two C_3 and eventually C_2 molecules.

Two considerations, atom economy and volumetric productivity, are paramount as one evaluates the metabolic possibilities summarized in **Figure 2**. Stoichiometrically, any chemical transformation that involves CO_2 release (e.g., conversion of pyruvate to ethanol) represents an inherent source of inefficiency. Thus, whereas the maximum theoretical yield of ethanol from glucose cannot exceed 67% (carbon mole basis), the yield of 3-methyl-1-butanol can be higher. Volumetric productivity (typically measured as grams product synthesized per liter fermentor volume per hour) is an important indicator of the efficiency with which the manufacturing plant is utilized and profoundly impacts the overall economics of the bioprocess. For example, the volumetric productivity of a state-of-the-art fermentation process that converts sugar into ethanol exceeds 1.6 g L^{-1} h^{-1} (59), whereas the highest reported productivity of fatty acids and derivatives is less than 0.2 g L^{-1} h^{-1} (48, 74). These quantitative factors must be borne in mind as one contemplates the possibilities and constraints associated with the biofuels described below.

ETHANOL

Although ethanol is not an ideal fuel molecule owing to its corrosive and highly hygroscopic nature and low energy content, it has already been commercialized as a biofuel and will therefore continue to influence the energy economy and the environment until it is replaced by a better alternative. *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Pichia stipitis* are the most widely used microbes to produce ethanol by fermentation, although alternative hosts are also being developed, including *Clostridia* and *E. coli*, to ameliorate some of the most significant problems with existing processes. In this section, we focus on the potential use of *E. coli* as a biocatalyst for ethanol fermentation.

FAME: fatty acid methyl ester

E. coli is naturally capable of converting sugars into ethanol via a heterofermentative process. Under anaerobic conditions, pyruvate is converted into acetyl-CoA and formate by pyruvate formate lyase (Pfl). Acetyl-CoA is reduced to ethanol with the concomitant consumption of two molecules of NADH in a reaction catalyzed by a bifunctional ethanol dehydrogenase (AdhE) (42, 69) (**Figure 3**). However, this native pathway is suboptimal because each mole of ethanol is accompanied by the synthesis of one mole of acetate and two moles of formate. Thus, its maximum theoretical yield is 0.26 g ethanol g^{-1} glucose, whereas the maximum theoretical yield of a homofermentative pathway is 0.51 g ethanol g^{-1} glucose (36). Upon knockout of the native *pflB* gene and introduction of heterologous *pdc* and *adhB* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II, respectively, from *Z. mobilis*, an engineered *E. coli* strain was able to generate ethanol as its primary fermentative product (34) (**Figure 3**). To further enhance its ethanol producing ability, the endogenous *frd* genes were also deleted to eliminate succinate production. The resulting mutant produced 45 g L^{-1} ethanol over 72 h in a complex growth medium with a carbon yield of 0.45 g ethanol g^{-1} glucose (i.e., nearly 90% theoretical maximum) (59). Its ethanol tolerance could be further improved via a three-month

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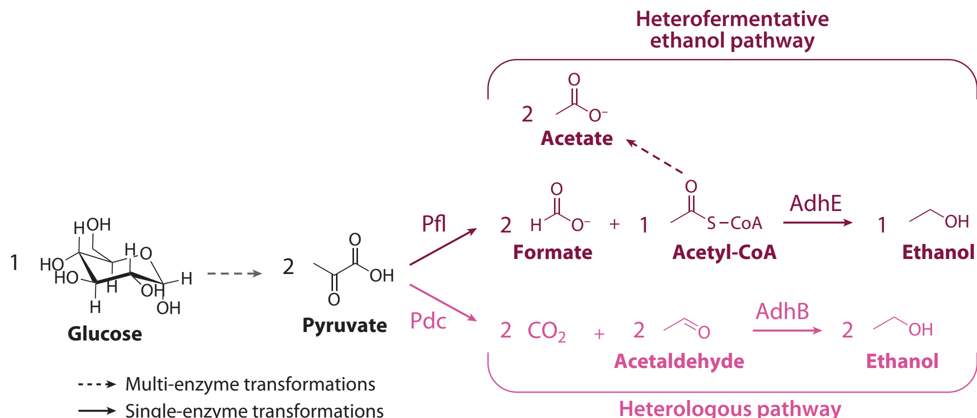

Figure 3

The heterofermentative ethanol pathway found naturally in *Escherichia coli* and the heterologous pathway from *Zymomonas mobilis*. In the native pathway, one mole of glucose is converted into two moles of formate, one mole of acetate, and one mole of ethanol. In the engineered pathway, one mole of glucose is converted into two moles of ethanol and two moles of carbon dioxide. Pfl, pyruvate formate lyase; AdhE, alcohol dehydrogenase; Pdc, pyruvate decarboxylase; AdhB, alcohol dehydrogenase II.

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directed evolution experiment involving alternate cycles of selection in liquid media for increased ethanol tolerance and selection on solid media for increased ethanol production (87). A series of additional genetic engineering steps enabled further development of a strain of *E. coli* that rapidly grew and produced alcohol in minimal medium (89). The state-of-the-art strain produced 46 g L^{-1} ethanol over 72 h in minimal medium containing a mixture of C_5 sugars and betaine, and had a carbon yield of 0.5 g ethanol g^{-1} xylose (36, 88). Not only did this *E. coli* strain enable utilization of a lower-grade carbon source (xylose is the principal product of hemicellulose hydrolysis), its atom economy was higher than any previously reported ethanologenic biocatalyst.

Notwithstanding these impressive technical achievements, commercial ethanol fermentation by *E. coli* requires tackling at least two additional challenges. First, cellulosic and hemicellulosic hydrolysates, which are the preferred raw material for biofuel production, contain organic acids, furan derivatives, and phenolic compounds that inhibit the growth of *E. coli* more so than *S. cerevisiae*, for example. This problem transcends ethanol and is relevant to the potential utility of *E. coli* as a biocatalyst for all other types of energy-rich molecules. Although improved pretreatment of the cellulosic feedstock is being considered, genetic engineering and/or selection is a more attractive strategy for addressing this problem (43, 57). Second, the high sugar concentration in the fermentation medium results in osmotic stress, a problem that can be mitigated with osmolyte supplementation (e.g., betaine, trehalose, proline, and/or glutamate), albeit at additional cost (36, 40, 81).

ISOPROPANOL AND BUTANOL

Nearly one hundred years ago, in response to the high demand for acetone during World War I, a fermentation process for acetone production was commercialized in Germany using *Clostridium acetobutylicum* as the biocatalyst. Because its side products, isopropanol and n-butanol, have better properties than ethanol as biofuels, the goal of producing such a mixed biofuel has attracted attention. As genetic tools are limited in *Clostridium*, it is also of interest to introduce the native pathway into *E. coli*. In 1998, the genes from *C. acetobutylicum* ATCC

824 encoding acetone biosynthesis, including acetyl-CoA acetyltransferase (*thl*), acetoacetyl-CoA transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*), were functionally coexpressed in *E. coli*, giving rise to a recombinant host that produced 5 g L^{-1} acetone with a volumetric productivity of 0.5 g L^{-1} h^{-1} (10). To convert acetone into isopropanol in *E. coli*, the *adh* gene encoding an alcohol dehydrogenase from *Clostridium beijerinckii* was synthesized with codon optimization. The benchmark *E. coli* strain harbored the *thl* gene from *C. acetobutylicum*, *atoAD* genes from *E. coli* (encoding both subunits of the acetyl-CoA:acetoacetyl-CoA transferase), *adc* from *C. acetobutylicum*, and *adh* from *C. beijerinckii*, and produced 5 g L^{-1} isopropanol with a volumetric productivity of 0.4 g L^{-1} h^{-1} (**Figure 4**) (27); both parameters were higher than native *Clostridium* strains. The engineered strain also achieved 44% of the theoretical maximum carbon yield (0.33 g isopropanol g^{-1} glucose). However, a homofermentative pathway that converts glucose into isopropanol is not redox balanced. Oxidative conversion of glucose to acetyl-CoA yields four moles of NADH per mole glucose, whereas production of each mole of isopropanol consumes only one mole NADPH (**Figure 4**).

For *n*-butanol production in *E. coli*, the native biosynthetic pathway from *C. acetobutylicum*, encoded by the *thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE2* genes, was introduced into this heterologous host (**Figure 4**). However, the resulting recombinant strain only produced

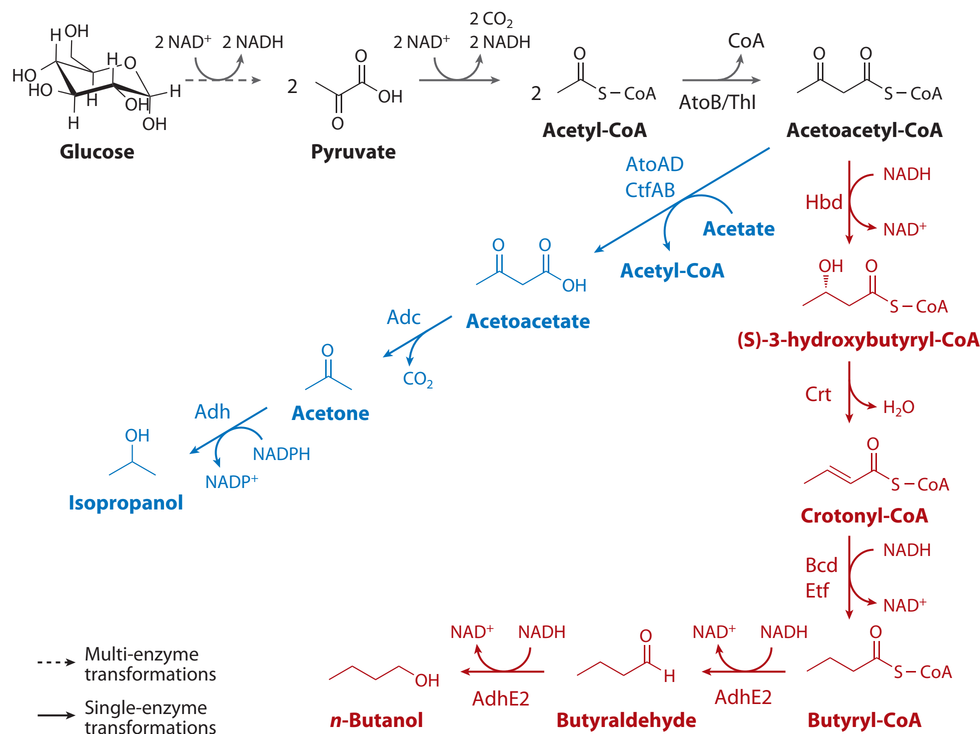

Figure 4

Metabolic pathways for isopropanol (*blue*) and *n*-butanol (*red*) production in engineered *Escherichia coli*. AtoB/Thl, acetyl-CoA acetyltransferase; AtoCD/CtfAB, acteoacetyl-CoA transferase; Adc, acetoacetate decarboxylase; Adh, alcohol dehydrogenase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; AdhE2, aldehyde/alcohol dehydrogenase.

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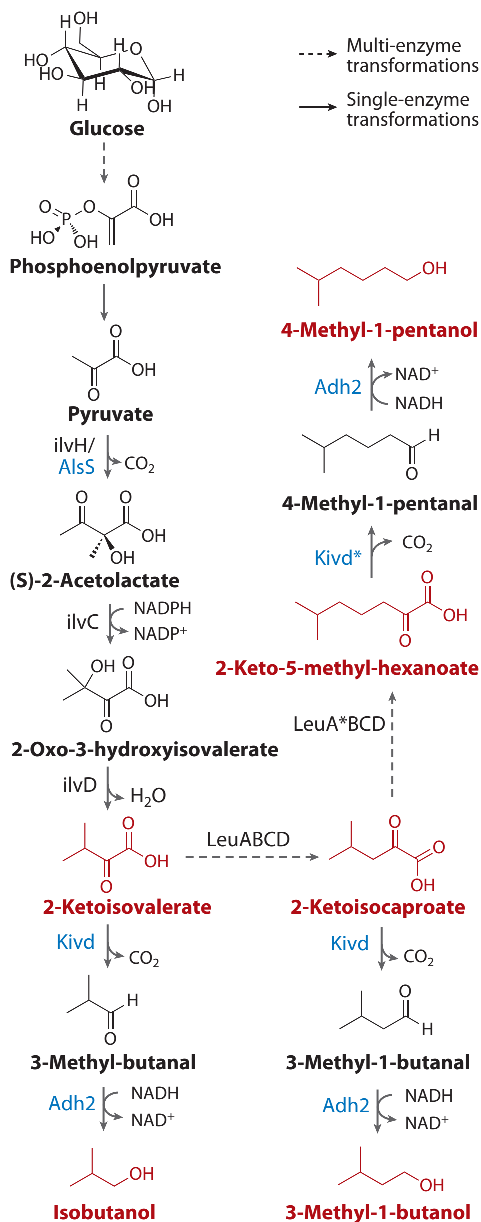
13.9 mg L^{-1} butanol under anaerobic conditions using glucose as the carbon source (4). To improve the yield and productivity of *n*-butanol, alternative enzymes from other microorganisms were evaluated. When the *E. coli atoB* gene was introduced in place of the *C. acetobutylicum thl* gene, butanol productivity increased more than threefold (4). Additionally, competing host pathways for carbon and reducing equivalents were deleted. The best strain harbored deletions in the *ldhA*, *adhE*, *frdBC*, *pta*, and *fnr* genes, and therefore produced markedly lower quantities of acetate, lactate, ethanol, and succinate. The highest butanol productivity observed in *E. coli* was 1.2 g L^{-1} over a 60-h period, with a carbon yield that was 15% of the theoretical maximum of 0.41 g butanol g^{-1} glucose (35). Although the latter value is significantly lower than in native *Clostridia*, it represents a promising starting point for further engineering.



OTHER SHORT-CHAIN ALCOHOLS

Short-chain alcohols can also be accessed in *E. coli* from 2-ketoacids, common intermediates in amino acid biosynthetic pathways. Two enzymes, 2-ketoacid decarboxylase (KDC, encoded by the *kivd* gene) from *Lactococcus lactis* and alcohol dehydrogenase (ADH, encoded by the *adh2* gene) from *S. cerevisiae*, have broad substrate specificity toward intracellular 2-ketoacids (**Figure 5**). When the corresponding genes were expressed in *E. coli*, six short-chain alcohols including 1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol were produced (5). The productivity of an individual alcohol correlates with the level of its corresponding ketoacid. For example, overexpression of *alsS* from *Bacillus subtilis* and *ilvCD* from

Figure 5

Nonfermentative pathways for production of short-chain alcohols in *Escherichia coli*. Pathways yielding isobutanol, 3-methyl-1-butanol, and 4-methyl-1-pentanol are shown as examples. The 2-ketoacids and short-chain alcohols are in red, and the heterologous enzymes are labeled in blue. Kivd, ketoacid decarboxylase; Adh2, alcohol dehydrogenase; ilvH/AlsS, acetolactate synthase; ilvC, acetohydroxy acid isomeroreductase; ilvD, dihydroxy acid dehydratase; LeuA, 2-isopropylmalate synthase; LeuCD, 2-isopropylmalate isomerase; LeuB, 3-isopropylmalate dehydrogenase. LeuA* and Kivd* represent the engineered enzymes.

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E. coli results in accumulation of 2-ketoisovalerate; when *kivd* and *adh2* are coexpressed in this host, the resulting *E. coli* strain produced 22 g L^{-1} isobutanol over 110 h with a carbon molar yield of 86% of the theoretical maximum (**Figure 5**) (5). This pathway has also been introduced into *Synechococcus elongatus* PCC7942. When expression of the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) gene was enhanced in conjunction with introduction of this pathway, the resulting bacterium photoautotrophically converts carbon dioxide into isobutyraldehyde and isobutanol (6). Productivity of other alcohols, including *n*-butanol, *n*-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and (R,R)-2,3-butanediol in *E. coli*, has also been enhanced via a combination of metabolic and protein engineering (7–8, 13, 18–19, 72, 85–86).

Although wild-type *E. coli* cannot produce 2-ketoacid intermediates corresponding to alcohols longer than C_6, C_6–C_8 alcohols have been derived from the isoleucine biosynthesis pathway in an engineered strain of *E. coli*. To do so, 2-isopropylmalate synthase (LeuA) was modified by structure-based mutagenesis to enlarge its substrate-binding pocket. The resulting long-chain ketoacids were converted into C_5–C_8 alcohols by a mutant of the *L. lactis* KDC (**Figure 5**) (92).

To improve the tolerance of *E. coli* toward alcohols under aerobic conditions, a combination of genome-wide transcriptional analysis, genetic knockouts, and network component analysis was employed. A malfunction in the quinone/quinol cycle was observed in the presence of elevated isobutanol levels. The *n*-butanol and isobutanol responses were qualitatively similar, whereas ethanol altered the expression of *pspABCDE* and *ndh*, whose gene products manage the proton motive force of *E. coli* (12).

ISOPRENOIDS

Both the nonmevalonate and the mevalonate pathways for isoprenoid biosynthesis have been successfully engineered in *E. coli* (2, 62). The theoretical carbon yields of C_5 building blocks from the two pathways are 0.29 and 0.25 (66), respectively. In turn, these building blocks have been converted into value-added chemicals. For example, amorpha-4,11-diene, a precursor of the antimalarial drug artemisinin, has been produced at levels up to 25 g L^{-1} over 120 h in *E. coli* (80). Fuel-like isoprenyl alcohols or isoprenes can also be synthesized from isopentenyl pyrophosphate (IPP), dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP), or farnesyl pyrophosphate (FPP) in the presence of phosphatases or pyrophosphatases. For example, the isoprene synthase gene (*ispS*) from poplar was introduced into *E. coli* to convert DMAPP to isoprene (56, 71). Similarly, *nudF* from *B. subtilis* has also been cloned into *E. coli* to convert IPP to isopentenol (84). The potential of these pathways with respect to atom economy or volumetric productivity remains to be explored.

IPP: isopentenyl pyrophosphate

DMAPP: dimethylallyl pyrophosphate

GPP: geranyl pyrophosphate

FPP: farnesyl pyrophosphate

FATTY ACID DERIVATIVES

Due to their high energy density and low water solubility, fatty acids are arguably the most appropriate biofuel precursors in a cell. Moreover, as commercial biodiesel is already a fatty acid–derived product, it provides a benchmark for microbial biofuels obtained via the fatty acid biosynthetic pathway. Conceptually, the challenge of overproducing fatty acid–derived biofuels in *E. coli* can be separated into two problems. First, productivity of the tightly regulated fatty acid biosynthetic pathway must be enhanced to yield a fatty acid mixture with an acceptable distribution of chain length and degree of unsaturation. The theoretical maximum carbon yield of this process is in the range of 0.29–0.35 g fatty acid g^{-1} glucose (66). Second, the spectrum of fatty acids thus produced must be chemically or enzymatically tailored into a fuel-like substance.

Fatty acid biosynthesis and its regulation have been extensively investigated in *E. coli* (26, 51, 83) (**Figure 6**). The primary use for fatty acids in this bacterium is in phospholipid biosynthesis; to do so, the fatty acyl moiety is

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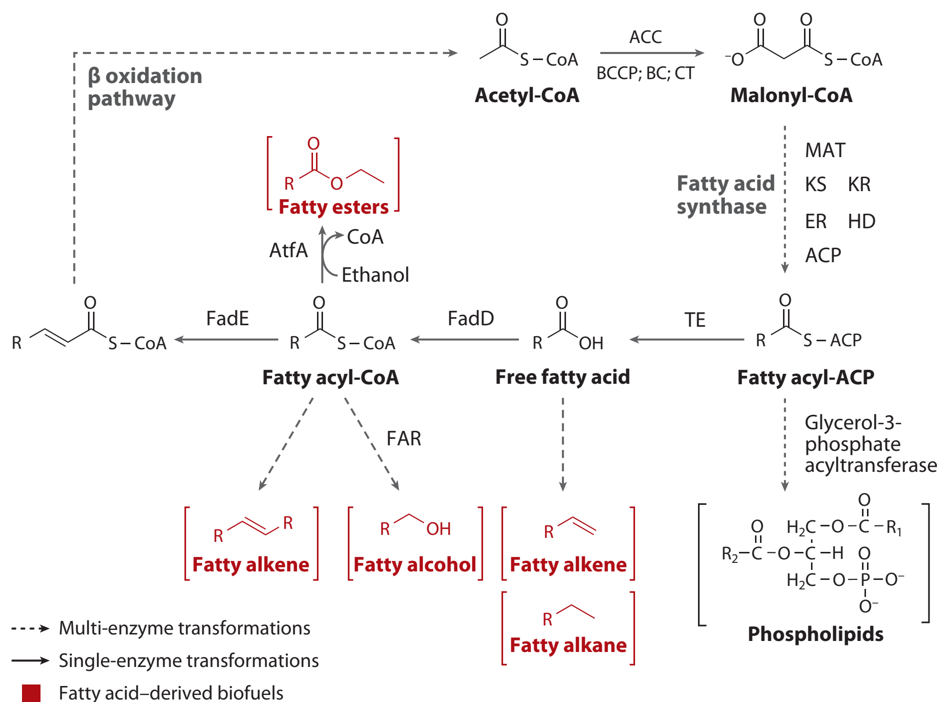

Figure 6

Fatty acid biosynthesis in *Escherichia coli* and fatty acid–derived biofuels. BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyltransferase α, β-subunits; MAT, malonyl-CoA:ACP transacylase; KS, β-ketoacyl-ACP synthase; KR, β-ketoacyl-ACP reductase; HD, β-hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase; ACP, acyl carrier protein; TE, thioesterase; FadD, fatty acyl-CoA synthetase; FadE, fatty acyl-CoA dehydrogenase; AtfA, wax ester synthase; FAR, fatty acyl-CoA reductase; R, long-carbon chain.

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directly transferred from the acyl carrier protein (ACP) onto glycerol derivatives by glycerol-3-phosphate acyltransferase. Smaller quantities of fatty acyl-ACP are also converted into lipid A, lipoic acid, and other minor products in *E. coli*. Excess fatty acids are rapidly degraded by activation as CoA thioesters, followed by oxidative cleavage into acetyl-CoA. Overall, fatty acid biosynthesis is tightly regulated at multiple levels. Transcription of the fatty acid regulon is controlled by FadR and FabR (26). Additionally, acetyl-CoA carboxylase (ACC) (the first dedicated enzyme in fatty acid biosynthesis) and at least two components, FabH and FabI, of the fatty acid synthase are feedback inhibited by long-chain fatty acyl-ACPs (20, 29, 30). Together, these mechanisms ensure that the cell does not accumulate excess quantities of this energy-rich hydrocarbon.

ACC: acetyl-CoA carboxylase

To overproduce fatty acids in *E. coli*, four genetic changes have been introduced into its genome. First, the *fadD* gene (encoding the fatty acyl-CoA synthetase) was deleted to prevent degradation of the desired product. Second, *E. coli* ACC was overexpressed to increase the supply of malonyl-CoA. Third, *E. coli* thioesterase was overexpressed to attenuate the ability of fatty acyl-ACPs to inhibit product formation. And lastly, a plant thioesterase with medium-chain length specificity was expressed in *E. coli* to increase the yield of shorter-chain fatty acids (49). With additional optimization, the resulting strain of *E. coli* produced 4.5 g L^{-1} day^{-1} fatty acids

in minimal medium with a carbon molar yield that was 20% of the theoretical maximum (48). Two other independent studies have verified the benefits derived from such changes. In one case, an alternative plant acyl-ACP thioesterase (BTE) was expressed in *E. coli* to replace the activity of the native thioesterase (47). The resulting strain produced comparable levels of fatty acids. In another study, the *fadE* gene (encoding an acyl-CoA dehydrogenase) was deleted along with overexpression of the endogenous thioesterase (74). The resulting strain produced 1.2 g L^{-1} fatty acids in shake-flask cultures with a carbon yield of 14% of the theoretical maximum (74).

In an effort to systematically identify and quantitatively understand all the factors that control carbon flux from glucose to fatty acids, a cell-free system has been developed that contains physiologically accurate ratios of all the macromolecular components responsible for transforming acetyl-CoA into fatty acids in *E. coli*. Already, this approach has started to provide useful direction for future genetic engineering. For example, the strong dependence of fatty acid synthesis on malonyl-CoA availability was quantified, highlighting the value of yet further enhancement of ACC activity in vivo. In contrast, cell-free analysis showed that NADPH was unlikely to be limiting under most physiological conditions, but when that happens, low expression of a phosphite dehydrogenase mutant could efficiently interconvert NADH and NADPH. Cell-free analysis also identified components that influence fatty acid productivity in a nonlinear fashion. For example, sharp maxima were observed in the dependence of fatty acid biosynthetic rates on thioesterase or ACP concentrations, suggesting that the in vivo concentrations of both proteins must be carefully controlled in order to optimize fatty acid productivity (48) (**Figure 7**).

A variety of chemoenzymatic strategies have been explored for converting overproduced fatty acids in *E. coli* into biofuels (**Figure 6**). For example, a broad specificity acyltransferase

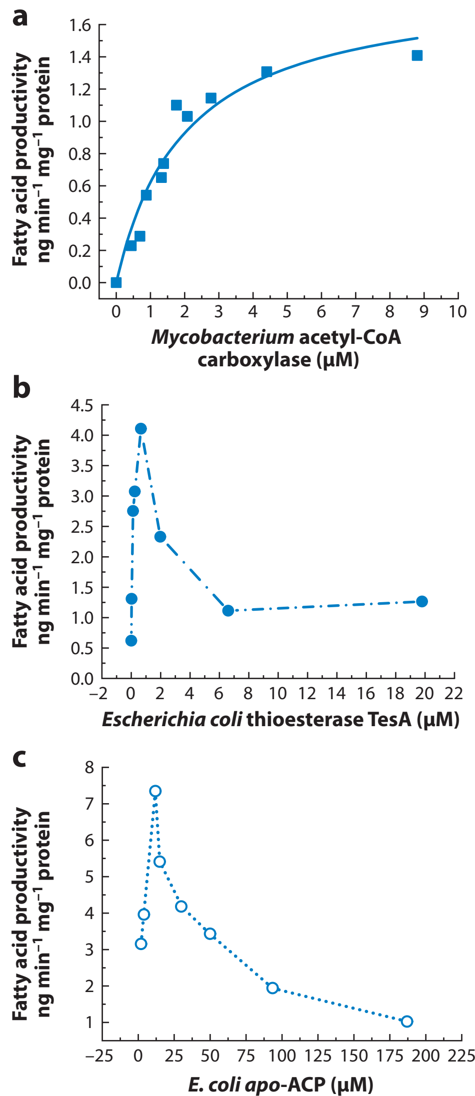

Figure 7

Evidence for optimal concentration of key proteins involved in fatty acid biosynthesis in *Escherichia coli*. Cell-free systems were used to assess the dependence of fatty acid productivity on (*a*) acetyl-CoA carboxylase (ACC), (*b*) TesA thioesterase, and (*c*) acyl carrier protein (ACP) concentrations. Whereas increasing ACC concentration leads to a saturable increase in the rate of fatty acid production, TesA and ACP exhibit sharp maxima, implying that their in vivo activities must be carefully optimized.

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FAEE: fatty acid ethyl ester

(AtfA) from *Acinetobacter baylyi* ADP1 was identified that transesterified fatty acyl-CoA intermediates with ethanol (38, 77). This discovery has opened the door to directly biosynthesize fatty acid ethyl esters (FAEE) (39, 78), a close structural analog of commercial biodiesel, in *E. coli*. Indeed, this has been recently achieved in a single strain of *E. coli* by combining the *fadE* deletion, thioesterase overexpression, overexpression of a mutant *fadD* (M335I) gene for fatty acyl-CoA production, overexpression of *pdc* and *adhB* for ethanol production, and overexpression of the *atfA* gene for the final ligation. The resulting mutant produced 0.7 g/L FAEE, with a carbon yield that was 9.4% of the theoretical maximum (74).

Fatty acids can also be reduced to the corresponding fatty alcohols to generate a more usable biofuel. All known fatty acid reductases (FARs) are membrane-associated NADPH-specific enzymes (16, 54, 63, 65). We have coexpressed the jojoba FAR gene in *E. coli* to obtain approximately 0.2 g L^{-1} C_{12}–C_{18} fatty alcohol over a 20 h period (T. Liu and C. Khosla, unpublished results). Independently, five *Arabidopsis* fatty acyl-CoA reductase genes have been functionally expressed in *E. coli*, although very low fatty alcohol titers were reported from these recombinant hosts (22). Also, the FAR gene *acr1* (AAC45217) from *Acinetobacter calcoaceticus* was used to produce approximately 0.1 g L^{-1} medium-chain fatty alcohols in *E. coli* (74). The discovery or engineering of improved FAR enzymes promises to be a fruitful direction for future research.

The ideal end product of a fermentation process that overproduces fatty acids would be an alkane mixture with appropriate properties for use as diesel or jet fuel. Whereas some algae, plants, and fungi are known to produce alkanes from fatty acids, the genes involved in these biotransformations are unknown (15, 21, 55, 70, 79). On the other hand, alkenes can be synthesized in *E. coli* by head-to-head condensation of two fatty acyl-CoAs or decarboxylation of free fatty acids. The genes involved in these metabolic transformations have been cloned and functionally expressed in *E. coli*, although the observed productivities were low (1, 9, 25). Alternatively, chemical processes can also be envisaged for the inexpensive transformation of free fatty acids into alkanes. For example, fatty acid mixtures in crude extracts of *E. coli* fermentations have been decarboxylated into saturated alkanes using a Pd/C catalyst (47).

SUMMARY POINTS

1. Engineered *E. coli* strains can ferment a variety of sugars into ethanol as the predominant product. Although its yield and productivity can be high, other technical barriers need to be overcome in order for this process to become economically viable.
2. *E. coli* can also be engineered to efficiently ferment sugars into isopropanol using genes from *Clostridium*. However, more reduced products such as ethanol and butanol must be concomitantly produced in order for the overall fermentation process to be redox balanced. Efficient butanol production in *E. coli* remains a significant challenge.
3. By introducing a 2-ketoacid decarboxylase and an alcohol dehydrogenase into *E. coli*, a variety of other short-chain alcohols can be produced from fermentable sugars. These compounds are superior to ethanol as fuels, and the atom economy and volumetric productivity of such processes are rapidly approaching those of benchmark ethanol fermentations.

4. Fatty acid biosynthesis and possibly isoprenoid biosynthesis can be harnessed in *E. coli* to produce hydrocarbons that are virtually indistinguishable from mainstream transportation fuels. Early data regarding carbon yields and productivities of these processes highlight the elaborate regulatory controls associated with these metabolic pathways, but also point to significant potential for further improvement via engineering.

FUTURE ISSUES

1. Compared with the extraordinary atom economy and volumetric productivity of a modern refinery that transforms crude oil into transportation fuels, the design of a biorefinery that converts cheap lignocellulosic biomass into fuel is in its infancy. As of now, the choice of the optimal biofuel is unclear, nor is it obvious that a single product type will dominate the growing biofuel market in the future.
2. Regardless of the product choice, a deep, quantitative understanding of the underlying metabolism is essential for engineering an efficient and environmentally friendly biocatalyst and biorefinery. Whereas a wealth of qualitative information is available regarding the metabolism of *E. coli*, quantitative data (as well as tools to rapidly generate and refine such data) are lacking. Studies with cell-free systems may prove useful in this regard, as they enable direct probing of the relevant metabolic processes in a rapid, less-constrained and quantitative fashion.
3. Independent of the challenge of engineering potent biocatalysts that convert sugars into fuels, upstream problems such as converting lignocellulosic biomass into fermentable carbon and downstream problems such as improving biocatalyst tolerance to high fuel concentrations will require attention, if microbial approaches to greenhouse gas reduction are to be successful.

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DISCLOSURE STATEMENT

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