

Expanding the repertoire of biofuel alternatives through metabolic pathway evolution

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A multitude of concerns that include climate change, political instability, and depletion of petroleum resources has recently ignited renewed interest in fossil fuel alternatives (1). As a result, microbial systems have been extensively explored and successfully used for the biosynthesis of some biofuels, most notably ethanol (2, 3). Higher-chain alcohols, however, offer several advantages compared with ethanol, such as higher energy density and lower water solubility (4). Despite this, the biosynthesis of such alcohols remains a daunting task, with the possible exception of 1-butanol. A new paradigm is now emerging, however, as evidenced in a recent issue of PNAS by the work of Liao and coworkers at University of California, Los Angeles (5). Their work demonstrates the construction of nonnatural metabolism that allows the biosynthesis, for the first time, of an array of alcohols not readily produced by microorganisms. In the past, metabolic engineering efforts have exclusively focused on rewiring native metabolic pathways toward a metabolic pathway of interest (6). At the same time, protein engineering, in general, and directed evolution, in particular, have mainly focused on the improvement of single-protein functions (7) with few examples of creating single proteins with novel functions (8, 9). The high evolutionary potential of biosynthetic enzymes has therefore remained largely untapped and their substrate promiscuity has been exploited for the generation of novel compounds mainly through feeding experiments (10, 11).

Recently, Liao's group demonstrated the efficient production of C4 and C5 alcohols for the first time in an *Escherichia coli* recombinant strain (4) overexpressing native metabolic pathways together with 2 heterologous enzymes. As an extension to this study, the work by Zhang *et al.* (5) focuses on the biosynthesis of a number of higher-chain alcohols (>C5), namely 1-propanol, isobutyl alcohol, 1-butanol, (*S*)-2-methyl-1-butanol, 3-methyl-1-butanol, 1-pentanol, 4-methyl-1-pentanol, (*S*)-3-methyl-1-pentanol, 1-hexanol, (*S*)-4-methyl-1-hexanol, and (*S*)-5-methyl-1-heptanol. The biosynthesis of such an extensive and remarkable array of alcohols, currently produced only through the Ehrlich pathway, was achieved by

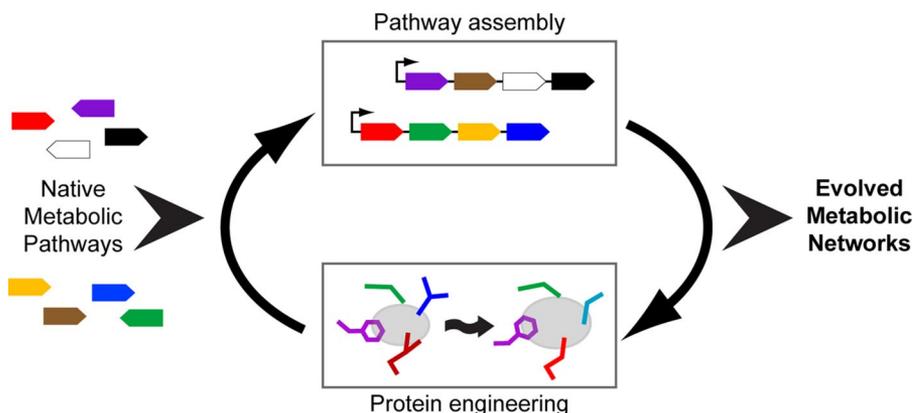


Fig. 1. Evolution of a metabolic network through a combination of protein and metabolic engineering.

expanding existing metabolic networks of the host microorganism through a combination of protein engineering and metabolic engineering approaches (Fig. 1); thus, the authors accomplished not only the expansion of the metabolic capabilities of the cell, but also the efficient production of novel biofuels. More specifically, the report by Zhang *et al.* addresses a critical question in metabolic engineering: can we rewire old enzymes to create new metabolic pathways? In the present work, this task was achieved through the addition of 2 heterologous enzymes, 2-ketoisovalerate decarboxylase (KIVD) from *Lactococcus lactis* and alcohol dehydrogenase from *Saccharomyces cerevisiae* together with the overexpression of a feedback-insensitive L-threonine biosynthetic pathway and parts of the L-valine and L-leucine pathways. The metabolic efficiency of the resulting artificial pathway, capable of producing C6 alcohols, was next enhanced through targeted mutations of KIVD that enhanced its specificity toward 2-keto-4-methylhexanoate, the key precursor of the C6 alcohol 3-methyl-1-pentanol. Enlargement of the binding pocket of another critical enzyme in the artificial pathway, 2-isopropylmalate synthase (LeuA) resulted in another 2-fold increase in overall production of 2-keto-4-methylhexanoate. Although additional mutations of LeuA did not result in any further production enhancement, they did, however, result in the biosynthesis of several other alcohols, including two C7 and one C8 alcohol for the first time.

The work of Zhang *et al.* (5) provides a new paradigm in the area of biofuel synthesis because it describes the vast potential of evolving a metabolic pathway from the ground up and is certainly the first important step toward the application of longer-chain alcohols as biofuels. It is important to note that, until now, the proposed route for the biosynthesis of a very limited number of long-chain alcohols has been through the more complicated isoprenoid biosynthetic pathway (12). Furthermore, the long-chain alcohols produced through the presented approach could also provide new alternatives as renewable chemical agents in several other applications. However, a number of challenges lie ahead. For example, the properties of the produced alcohols remain to be assessed to determine their suitability as fuel molecules. Furthermore, it will be necessary to further enhance production yields in order for these alcohols to become competitive alternatives to fossil fuels. Such enhancement will require, among other things, an enhanced availability of metabolic cofactors required in the engineered pathways, including reducing agents and CoA esters (13, 14). Finally, the robustness of the engineered strains

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can become an important challenge, especially during process scale-up. It is to be expected that most of these challenges will be addressed in the future through a combination of approaches from systems biology, directed evolution, and synthetic biology (15, 16).

Ultimately, the feasibility of the higher alcohols reported by Zhang *et al.* as biofuels will be determined by a web of technical, political, and socioeconomic factors, but the evolutionary strategy introduced will play a tremendous role in the progress of biocataly-

sis, in general, and metabolic engineering, in particular.

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