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Synthetic metabolism: metabolic engineering meets enzyme design

Tobias J Erb[1], Patrik R Jones[2] and Arren Bar-Even[3]

Metabolic engineering aims at modifying the endogenous metabolic network of an organism to harness it for a useful biotechnological task, for example, production of a value-added compound. Several levels of metabolic engineering can be defined and are the topic of this review. Basic 'copy, paste and fine-tuning' approaches are limited to the structure of naturally existing pathways. 'Mix and match' approaches freely recombine the repertoire of existing enzymes to create synthetic metabolic networks that are able to outcompete naturally evolved pathways or redirect flux toward non-natural products. The space of possible metabolic solution can be further increased through approaches including 'new enzyme reactions', which are engineered on the basis of known enzyme mechanisms. Finally, by considering completely 'novel enzyme chemistries' with *de novo* enzyme design, the limits of nature can be breached to derive the most advanced form of synthetic pathways. We discuss the challenges and promises associated with these different metabolic engineering approaches and illuminate how enzyme engineering is expected to take a prime role in synthetic metabolic engineering for biotechnology, chemical industry and agriculture of the future.

Addresses
[1] Biochemistry and Synthetic Biology of Microbial Metabolism Group, Max Planck Institute for Terrestrial Microbiology & LOEWE Research Center for Synthetic Microbiology (SYNMIKRO), Karl-von-Frisch-Str. 10, D-35043 Marburg, Germany
[2] Department of Life Sciences, Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, UK
[3] Systems and Synthetic Metabolism Group, Max Planck Institute for Molecular Plant Physiology, Potsdam-Golm, Am Mühlenberg 1, D-14476 Potsdam, Germany

Corresponding authors: Erb, Tobias J (toerb@mpi-marburg.mpg.de), Jones, Patrik R (p.jones@imperial.ac.uk)

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Introduction

The introduction of the concept of 'total synthesis' by Wöhler [1] was one of the milestones in chemistry [2]. The possibility to create non-natural compounds, color pigments, drugs, materials and catalysts from simple chemical building blocks catapulted chemistry into one of the key sciences of the 20th century and to a driving force of our modern world.

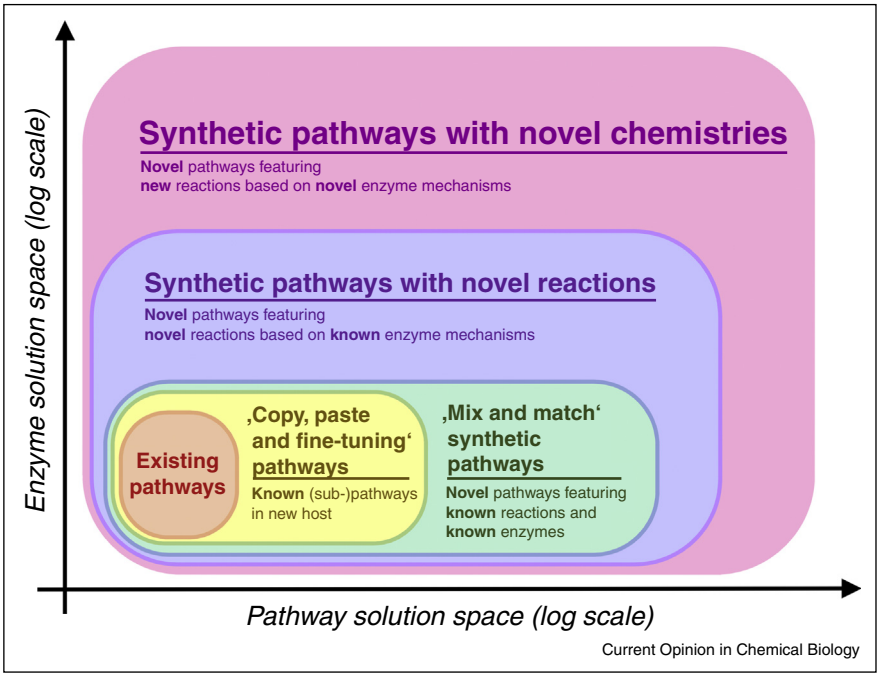
It has been one of the ultimate goals in biology to achieve the same conceptual and synthetic level as reached in chemistry, ever since the principle of 'metabolic engineering' was developed in the early 1990s. Yet, cells are still far from being 'little chemical factories' [3] and metabolic engineering has been limited in its synthetic capabilities so far, relying mainly on the transplanting known pathways to a new host followed by optimization.

To realize the full potential of metabolic engineering new strategies and approaches are required. Recent advances in molecular genetics, computational biology, and protein design open the chance to move metabolic engineering from a 'tinkering science' toward a truly synthetic discipline. Through combination of these approaches we will no longer be limited to existing pathways and enzymes but be able to design entirely novel pathways in a rational fashion and thereby realize synthetic metabolism. Here, we classify different metabolic engineering efforts according to the extent that they go beyond existing metabolic network structures. We provide an experimental roadmap toward achieving the synthetic metabolism goal, define the challenges and chances of *de novo* pathway design, and discuss possible future applications of synthetic metabolism.

Five levels of metabolic engineering

Several levels of metabolic engineering can be defined according to the synthetic character and the resulting biochemical solution space (Figure 1 and Table 1). Basic metabolic engineering efforts operate on the level of existing pathways within their natural host. Here, pathway productivity is improved only through gene deletions and overexpressions. In more advanced 'copy, paste and fine tuning' approaches, existing (sub-)pathways are introduced to another host, often a biotechnologically relevant strain, where they are eventually further modified by replacing individual enzymes. This results in relatively small changes to the overall metabolic structure supporting faster kinetics, an improved thermodynamic profile, or a more sustainable cofactor use. For example, in a pathway for *n*-butanol production, expressed in *E. coli*, substituting the thermodynamically limiting acetoacetyl-Coa synthetase with an irreversible acetyl-CoA:

Figure 1



The five different levels of metabolic engineering as defined in this review. The enzyme solution space describes the number of possible enzymes reactions available for a given strategy while the pathway solution space corresponds to the number of possible pathways that can be constructed. While level 1, 2 and 3 metabolic engineering efforts do not differ in enzyme solution space, because they all rely on known enzymes, level 4 and 5 metabolic engineering efforts provide new enzymes created through enzyme engineering or *de novo*-design.

malonyl-CoA acetyltransferase (decarboxylating) substantially improved product titers [4]. Such mild tinkering, however, does not alter the basic structure of the engineered pathways.

In contrast to above strategies that mainly build on existing pathways, 'mix and match' approaches expand the metabolic solution space. In these efforts, existing enzymes that are not known to work together in nature

Table 1

Definitions, features and examples for the five different levels of metabolic engineering.

	Definition	Typical feature	Example
Level 1	Optimize existing pathway in natural host	Knock out and overexpression of individual gene in natural host	Calvin cycle for CO_2-fixation: overexpresssion of transketolase to increase flux [61]
Level 2	Transfer and exchange of known (sub-)pathways in new host	Natural route replaced or modified with better - performing reactions	Transfer of Calvin cycle for CO_2-fixation: into *E. coli* to allow for sugar synthesis from CO_2 [62]
Level 3	Novel pathways created from known reactions	Non -natural route constructed from natural enzymes	MOG pathway for CO_2-fixation: recombination of known enzymes into a synthetic pathway [6]
Level 4	Novel pathways created from novel reactions that are based on known enzyme mechanisms	Non-natural route containing enzymes of modified substrate specificity	CETCH cycle for CO_2-fixation: recombination of known enzymes and engineered enzymes with new substrate and reaction specificities into a synthetic pathway [13]
Level 5	Novel pathways created from novel reactions that are based on novel enzymatic mechanisms	Non-natural route containing de novo designed enzymes	A CO_2-fixation cycle based on an not -yet developed Ni-Ga cofactor in an not-yet evolved artificial metalloprotein

are integrated into synthetic pathways to perform a given metabolic task with higher efficiency or novel functionality. Recent examples are a designer pathway for the biosynthesis of propane from glucose [5], a non-oxidative glycolysis for complete carbon conservation [6], or artificial C1 assimilation pathways that were designed by freely recombining existing enzyme reactions, even though these routes were not experimentally realized so far [6,7]. Such combinatorial design efforts can be automatized by software programs [8,9], though it is important to keep in mind that databases such as KEGG are not complete relative to the existing literature. To identify the most promising pathways out of many different possible routes, a comprehensive pathway analysis is recommended. Such an analysis compares different pathway candidates according to physicochemical properties, including resources consumption, thermodynamic feasibility, kinetic proficiency, toxicity and hydrophobicity of intermediates, as well as overlap with endogenous metabolism [10].

The main advantage of the 'mix and match' approach is that without the need to evolve novel reactions, a wide array of potential pathways can be identified. Yet, to fully tap into the (bio)chemical solution space, more synthetic metabolic engineering efforts aim at implementing pathways that include new reactions that are not known to exist in nature through enzyme engineering and *de novo*-enzyme design (see below). So far, only a handful of studies have implemented synthetic pathways that involve novel catalytic transformations. These rare examples include a synthetic route to 1,4-butanediol [11], a novel didanosine biosynthetic pathway [12] and a synthetic pathway for the fixation of carbon dioxide [13••]. These examples demonstrate how metabolic engineering can access novel products and non-natural pathways of improved efficiency with new reactions, paving the way toward truly 'synthetic metabolism', in which designer pathways are first drafted based on rational considerations. Only afterwards is the actual route realized experimentally by identifying, designing and recombining individual enzyme reactions.

Designer reactions for designer pathways

'New reactions' for synthetic pathways can be realized in several ways. One approach considers the backward reactions of enzymes that are mistakenly considered as irreversible, but which can actually sustain *in vivo* flux in both directions. While this approach is restricted to a small number of enzymes, it can offer a wide array of new options. For example, the 'irreversible' pyruvate formate-lyase was recently found to support formate assimilation *in vivo* [14], which allows multiple promising formate pathways to be established around this reaction [7]. Similarly, by acknowledging that the glycine cleavage system [15] is fully reversible *in vivo* [16], a new approach for carbon or formate assimilation could be developed [7].

Another way to find 'new reactions' is based on exploring and extending the substrate repertoire of existing enzymes. These strategies are promising if prospectives substrates are structurally related to the native substrates of known enzymes. In many enzyme superfamilies, only a limited amount of representatives have been experimentally characterized so far. This means that a sought enzyme activity might already naturally exist in an enzyme superfamily, only it was not discovered yet. For instance, the family of B12-dependent acyl-CoA mutases was for a long time only thought to consist of methylmalonyl-CoA mutase. However, very recently, enzymes-specific also for ethylmalonyl-CoA [17], isobutyryl-CoA, 2-hydroxybutyryl-CoA, and most recently isovaleryl-CoA [18] were identified. New tools like sequence similarity and genome neighborhood networks, as well as advanced structure prediction and docking tools will help to identify promising candidates to be tested experimentally [19,20•,21,22•,23].

Even if a sought reaction does not exist naturally, many enzymes are known to be intrinsically promiscuous, so that the reaction might be established (or 'extended') from a side-reaction of a given enzyme. Such promiscuous activity can provide an excellent starting point for further engineering to improve catalytic efficiency, especially when assisted by structural information [24]. Isovaleryl-CoA mutase activity was engineered from a side-reaction of isobutyryl-CoA mutase, providing novel options for the synthesis of branched C4 and C5 building blocks [18]. The naturally promiscuous CoA ligase matB was further engineered to feed novel substrates into polyketide biosynthetic assembly lines, giving rise to novel natural products [25,26]. Screening and structurally guided mutagenesis was used to provide carboxylating enoyl-CoA ester reductases (ECR) that can deliver novel building blocks for polyketide biosynthesis [27,28]. Promiscuous enzymes were also used to establish metabolic routes for the production of non-natural lactate-based polymers and esters in *Ralstonia eutropha* [29,30] and *Escherichia coli* [31,32]. Similarly, harnessing the intrinsic promiscuous catalytic potential of squalene hopene cyclases could provide new ways toward valuable cyclohexanoid monoterpenes if successfully integrated into metabolism [33].

Exploring the natural biochemical space and the promiscuity of enzymes will potentially cover many of the 'new reactions' required in synthetic pathway design. Yet, in some cases, it will be necessary also to design a required enzyme reaction *de novo*. An example is an artificial 'retro-aldolase' that was created through computational design and experimental evolution and can directly use acetone as donor in contrast to natural aldolases [34]. Even more progressive are efforts that give access to completely novel enzyme chemistries, which have not evolved in nature. A 'formolase enzyme' that could form the basis for a novel formaldehyde assimilation pathway was

conceived by computational design [35••]. The metathesis reaction that was exclusively used in synthetic chemistry so far was successfully functionalized for biology through the directed evolution of an artificial metalloenzyme [36•], which opens the chance to harness the principle of metathesis also for metabolic engineering. Very recently an enzyme that is catalyzes silicon-carbon bonds was evolved, providing a first step toward engineering the biotechnological production of organosilicon compounds [37•]. These pioneering studies provide exciting examples how the field of enzyme design will be able to provide many more novel catalysts for synthetic metabolism in future.

In silico-analysis of synthetic metabolic routes

For the *de novo* design of synthetic pathways it is essential to confirm that no thermodynamic or kinetic barriers are expected to constraint the activity of the new pathways. While it is difficult to obtain reliable estimation for the kinetics of yet to be evolved new reactions, the thermodynamic profile of a pathway can be calculated with rather high precision, even for pathways whose components are not fully defined yet [13••,38–40]. Such computational analyses will also need to take into account barriers that are generated by the sequential operation of several enzymes. Even though each reaction in a sequence might be thermodynamically feasible by itself, their sequential combination might lead to severe energetic barriers [40].

Another problem is the possibility of an overlap between the new pathway and endogenous metabolism. Such an overlap can result in an unregulated rewiring of cellular metabolic fluxes, which could have a deleterious effect on growth rate and yield. Alternatively, and equally problematic is the ability of endogenous metabolic fluxes to suppress the activity of synthetic pathways; especially if one of the pathway enzymes operates in the reverse direction under normal cell conditions. Uncontrolled drainage of pathway intermediates through other reactions, especially by enzymes in central carbon metabolism may also limit pathway activity. It is therefore important to analyze the compatibility of the novel pathways with endogenous metabolism before the *in vivo* implementation stage; this could be, at least partially, achieved by applying constrained-based modeling strategies, such as flux balance analysis [6].

Challenges during implementation of synthetic metabolism

One of the biggest experimental challenges in realizing synthetic metabolic routes is the recombination of enzymes from very different biological backgrounds with no common evolutionary and physiological history into one pathway. This could result in sub optimal kinetics that could substantially limit the efficiency of the novel pathways. Another major difficulty in expressing novel enzymes and pathways within a non-native host is the fact that enzymes in the cell are expected to face metabolites to which they were never exposed in their native metabolic context. This can result in a cross-inhibition of a given enzyme by the reaction products of other pathway enzymes, or cause undesired side reactions of a given enzymes with other pathway intermediates, resulting in the formation of inhibitory side-products or dead-end metabolites [13••,41]. Consequently, it is important to include appropriate mitigation strategies – 'hermeting strategies' (from *Hermes*, god of the travelers and crossroads) – into synthetic pathway design. Generally it can be anticipated that the more "synthetic" a pathway is, the more important it might become to include such strategies into pathway design.

An obvious strategy to minimize side reactions is to replace promiscuous enzymes with more specific isoenzymes or homologs. Alternatively, promiscuous enzyme can be improved by enzyme engineering to increase the discrimination factor between the desired reaction and an undesired side-reaction [13••]. Another solution is to apply 'metabolic proofreading'. This strategy includes the addition of auxiliary enzymes to the core sequence of a given synthetic pathway. These proofreading enzymes are not part of the actual pathway, but serve in removing toxic side products or recycling dead-end metabolites. Although metabolic proofreading and scavenging mechanisms apparently exist in naturally evolved pathways [42–47], these concepts have been largely neglected in synthetic metabolism design so far. However, through the combination of metabolic proofreading and enzyme redesign, the activity of a synthetic *in vitro* CO_2-fixation pathway was improved by more than an order of magnitude [13••]. Likewise, implementing a pathway for recycling the dead-end metabolite erythrose-4-phosphate was essential to establish an *in vitro* pathway for the conversion of glucose into polyhydroxybutyrate [48••].

Another approach to bypass deleterious overlap with central metabolism is to spatially confine synthetic pathways or parts thereof. Through the use of microcompartments pathway enzymes and intermediates can be insulated from the rest of cellular metabolism [49]. This approach has an additional advantage if a pathway intermediate is reactive and can damage the cellular machinery. Alternatively, synthetic protein scaffolds can keep pathway enzymes at near proximity, thereby increasing the effective concentration of intermediates [50,51].

Opportunities for synthetic metabolic pathways

Despite the challenges, there are many advantages for realizing synthetic metabolism. Generally this typically takes the form of a metabolic by-pass or the redirection of metabolic flux toward end-products that do not naturally accumulate in cultures of the chosen biotechnological

host. By freely combining existing and novel enzymatic reactions from various biological sources, optimal routes for a metabolic task can be drafted that allow to overcome any historical and ecological constraints of natural pathway evolution (*i.e.*, the serendipity of enzymes coming together in time and space). Combining an ACP-fatty acid thioesterase from plants with an engineered, chimeric monooxygenase allowed to establish a *de novo* pathway for ω-hydroxy octanoic acid production in *E. coli* [52]. In a bioretrosynthetic approach, a pathway for the production of non-natural compound didanosine was developed [12]. Finally, through metabolic retrosynthesis, artificial pathways for CO_2-fixation were drafted that are up to 30% more energy efficient compared to natural existing carbon fication routes, such as the Calvin cycle of plants, algae and cyanobacteria [6,13••].

While the implementation of non-natural, synthetic biochemical routes in living organisms poses a challenge because of potential interference with natural metabolism and the challenge of finding or engineering suitable catalysts, non-natural pathways also bear the chance that their intermediates can be completely decoupled from central metabolism and the genetic program of the cell. Thus, their implementation might actually prove easier from a physiological or regulatory point of view. In efforts to improve production of the terpenoid amorphadiene in *E. coli*, for instance, the transplantation of the mevalonate-isoprenoid route from yeast was more successful than classical engineering of the native *E. coli* deoxyxylulose 5-phosphate (DXP) isoprenoid pathway. Most probably, because the DXP pathway's native regulatory elements and feedback loops are deeply rooted in *E. coli* and could be circumvented by the non-native mevalonate-isoprenoid route that does not interact with the regulatory machinery of the cell [53].

Future applications for synthetic metabolic pathways

For a future sustainable economy that is independent of fossil carbon, the capture and utilization of CO_2 will be crucial [54]. Designer pathways for the optimal and direct conversion of CO_2 into value-added products or feedstocks could provide alternative solutions to conventional biomass production via photosynthesis [6,13••,41,55]. This also includes a reconsideration of current production processes in the chemical industry, which are still based on petrochemically-derived feedstocks, but might be shifted to and fueled by synthetic pathways in the future (*e.g.*, an extended formate or methanol metabolism [7,56,57]). Another challenge relates to agricultural productivity, which needs to be increased to feed a growing world population. So far, many efforts focused on improving natural existing CO_2-fixation pathways and enzymes in plants [58]. Through synthetic metabolism, however, novel options could be provided, such as synthetic CO_2-fixation pathways or photorespiration bypasses of higher efficiency [58–60]. Such novel solutions are currently explored by several laboratories and initiatives, including ours [6,13••,61,62•]. The future will tell whether synthetic metabolism can indeed provide viable solutions for these grand social, economic and environmental challenges of the future.

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