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Synthetic metabolism approaches: A valuable resource for systems biology

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Abstract

Synthetic biology modifies biological systems with the aim of creating new biological parts, devices, and even organisms. Systems biology deciphers the design principles of biological systems trying to derive the mathematical logic behind biological processes. Although different in their respective research approaches and questions, both disciplines are clearly interconnected. Without sufficient understanding of the biological system, synthetic biology studies cannot be properly designed and conducted. On the other hand, systems biology can profit from new biological systems generated by synthetic biology approaches, which can reveal important insights into cellular processes and allow a better understanding of the principles of life. In this article, we present state-of-the-art synthetic biology approaches that focus on the engineering of synthetic metabolism in microbial hosts and show how their implementation has led to new fundamental discoveries on enzyme reversibility, promiscuity, and "underground metabolism". We further discuss how the combination of rational engineering and adaptive laboratory evolution has enabled the generation of microbes with a synthetic central metabolism, leading to completely new metabolic phenotypes. These organisms provide a great resource for future studies to deepen our systems-level understanding on the principles that govern metabolic networks and evolution.

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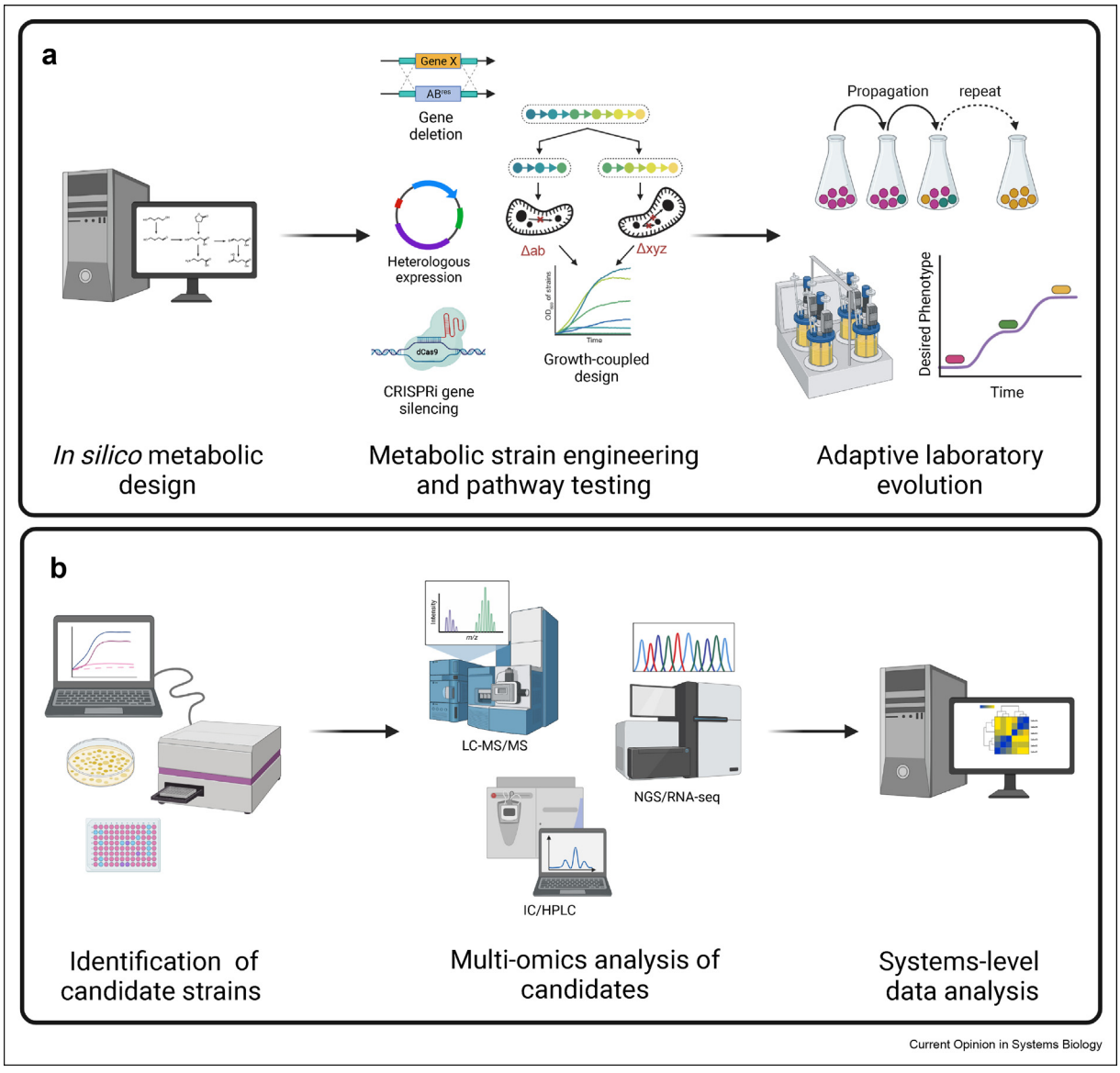
Introduction

In recent years, synthetic biology has matured as a discipline that can develop solutions to tackle some of the grand challenges of humanity. Hence, a major focus of current synthetic biology endeavors lays on sustainable bioproduction [1,2], reducing environmental pollution [3,4] and mitigating greenhouse gas emissions [5,6]. Many of these synthetic biology endeavors involve the modification of metabolism, traditionally referred to as metabolic engineering. In recent years this field has greatly advanced through novel genetic engineering tools and developed further towards the engineering of "synthetic" pathways. Some years ago, Erb et al. defined 5 levels of metabolic engineering, ranging from level 1,—the modification of existing pathways in their natural hosts to level 5,—the realization of synthetic pathways that include new-to-nature enzyme mechanisms [7]. Most current metabolic engineering approaches are on level 2,—transplantation of naturally existing metabolic pathways into non-native hosts, and level 3,—the mixing-and-matching of existing enzymes into new-to-nature pathways. These two levels of "synthetic metabolism" have already led to promising breakthroughs in recent years, in particular to the realization of new trophic modes and carbon or energy-conserving by-passes in *Escherichia coli* and several other microorganisms. The most spectacular recent advances in changing trophic modes include the conversion of the naturally heterotrophic *E. coli* to grow autotrophically on CO_2 and the reduced one-carbon (C_1) substrates methanol and formate [8,9]. The realization of these new trophic modes supports the stepwise transition from a sugar-based towards a more sustainable bio-economy that uses CO_2 or CO_2-derived C_1-compounds as feedstocks in biotechnological production [10,11].

Current synthetic metabolism studies, especially those conducted in well-characterized organisms, can employ a plethora of well-established genetic engineering methods [12]. Endogenous gene expression can be

Figure 1



From synthetic metabolism to systems biology. (a) In synthetic metabolism studies novel pathways are designed *in silico* before they are implemented into microbial hosts. A diverse toolkit enables genetic modification of the host genome and expression of pathway enzymes (e.g., gene deletion, heterologous expression, CRISPR interference). The concept of growth-coupled modular design relies on the creation of selection strains with a growth deficiency that can serve to test modules of a novel metabolic route (represented as colored dots connected by arrows). Enzymes of the respective module are expressed in the selection strain and growth of the strain indicates pathway activity. If rational engineering does not lead to sufficient activity of the novel metabolic route, adaptive laboratory evolution (ALE) can be employed for flux optimization. During ALE, a strain is cultivated under selective conditions for a prolonged time. The accumulation of beneficial mutations can lead to the emergence of intermediate subpopulations, but ultimately a dominant population with the desired phenotype is expected to overtake. **(b)** Engineered or evolved strains with a synthetic metabolism can serve as a valuable resource for systems biology. After the identification of interesting candidate strains, they should be analyzed for genomic modifications via next generation sequencing (NGS), for expression changes via NGS-based transcriptomics, for their metabolite levels and metabolic fluxes via quantitative proteomics, metabolomics and fluxomics approaches based on liquid chromatography mass spectrometry (LC-MS) and for their secretion profile via diverse chromatography methods (e.g., high pressure liquid chromatography (HPLC) or ion chromatography (IC)). Ultimately, the vast amount of data needs to be analyzed by systems biological approaches to derive novel insights to the logic of metabolism. Created with BioRender.com.

abolished (by gene deletion), reduced (e.g., by CRISPR interference, CRISPRi [13]) or increased (e.g., by exchanging the endogenous promoter or expressing the gene from a plasmid). Likewise, foreign genes can be introduced by heterologous gene expression either from a plasmid or from the genome. In the field of synthetic metabolism, the concept of growth-coupled design is beginning to receive increased attention (see Orsi et al. [14]). Within this approach the activity of a novel metabolic route is

coupled to the growth phenotype of the cell. To this end, selection strains that depend on the novel metabolic route for growth are designed *in silico* and created by gene deletions that lead to growth deficient phenotypes. Then, the enzymes of the novel route are expressed, and - if functional - the growth deficiency of the strain is released [15–18]. To facilitate testing and optimization, synthetic pathways are usually divided into modules that can be individually tested in different selection strains. While testing of individual modules via this approach often leads to growth, the rational introduction of an entire synthetic pathway often fails to lead to growth. If no or only limited growth via a module or the novel pathway can be achieved through rational interventions, adaptive laboratory evolution (ALE) presents a complementary system-wide engineering strategy to optimize pathway activity. Herein, the strain, expressing the pathway genes, is continuously cultivated under selective conditions to promote the emergence of beneficial, often nonintuitive, mutations that finally enable sufficient pathway activity [19] (Figure 1a). To date, a broad set of analytical tools enables the system-wide analysis of microbial strains harboring synthetic metabolism. After confirmation of pathway activity by growth tests and ^{13}C-carbon tracing (see Wenk et al. [15]), next generation sequencing (NGS) can be employed to detect genomic modifications resulting from evolution [20]. Analysis of transcript levels via RT-qPCR [9,21,22] or NGS-based transcriptomics [23,24] can reveal changes in gene expression. Finally, the enzyme and metabolite levels, as well as fluxes, can be analyzed via quantitative proteomics, metabolomics and fluxomics approaches based on LC-MS [25–28] (Figure 1b). Such omics approaches can provide in-depth information about systematic changes due to metabolic perturbations.

While the primary goal of most synthetic metabolism studies is clearly engineering related, the results of these studies often provide novel insights into the metabolic network of the host organism, which can be of high value for systems biology. In addition, some of the synthetic biology approaches used in engineering studies, such as growth-coupled testing of enzyme activities, can also be specifically employed to reveal fundamental insights in metabolism and metabolic networks.

In this article, we present an overview of state-of-the-art synthetic metabolism studies and their contributions to our understanding of metabolism (see Table 1). We discuss how relatively simple interventions led to the discovery of novel enzyme activities and underground metabolism. Furthermore, we focus on ALE as a tool to provide system-wide adaptations and discuss why ALE-derived strains with a synthetic metabolism present a valuable resource for systems biology explorations.

Synthetic biology approaches reveal *in vivo* enzyme activities and "underground" metabolism

In recent years, testing of enzymes in microbial selection strains has been increasingly used in synthetic metabolism studies [14]. In some cases, such strains have been used to study fundamental aspects of enzyme activities inside the cell, in other cases to reveal so-called underground metabolism.

One application of microbial selection strains is to solve long standing questions on *in vivo* enzymatic activities, such as their *in vivo* reversibility. While enzymes in theory catalyze reactions in both directions, in practice the direction they preferentially catalyze is determined by the cellular environment (e.g. by substrate, product and co-factor concentrations). The prediction of enzymatic catalysis *in vivo* can be very difficult as the exact physiological conditions within the cell are usually unknown and typically differ from the defined conditions in *in vitro* studies. Specific microbial selection strains can be designed to determine the catalytic feasibility of reactions *in vivo*, including the directionality of enzymatic catalysis. As an example, Zelcbuch et al. studied the enzyme pyruvate-formate lyase (Pfl), a central enzymatic node that converts pyruvate to acetyl-CoA and formate during anaerobic growth. This reaction was long assumed to only catalyze the cleavage of pyruvate to acetyl-CoA and formate in cellular conditions. However, by using a selection strain that depended on the reverse reaction of Pfl for growth, it was discovered that the enzyme can also catalyze the condensation of formate and acetyl-CoA *in vivo* [29]. With a similar growth-coupled approach, another study revealed that 6-phosphogluconate dehydrogenase (Gnd), which usually acts as a decarboxylase in the oxidative pentose phosphate pathway of *E. coli* is reversible *in vivo*. Using specific selection strains, it was shown that the enzyme can also catalyze the reaction in the carboxylating direction and potentially support a synthetic carbon fixation cycle, the GED (Gnd–Entner–Doudoroff) cycle [30]. Analyzing the strains adaptation to the new metabolic route, the authors gained insights into the flexibility of central metabolism and showed how the reversibility of enzymes can be used for engineering novel pathways.

While these two studies analyzed the reversibility of central metabolic enzymes, other recent studies used growth-coupled selection to show the *in vivo* ability of certain enzymes to catalyze side-activities of their canonical activity (promiscuous activities). Using a selection strain auxotroph for serine biosynthesis, He et al. showed that the low-specificity threonine aldolase (LtaE) of *E. coli*, which usually cleaves threonine into glycine and acetaldehyde can also condense glycine and formaldehyde into serine *in vivo* [31]. In the same study,

Table 1

Selection of synthetic biology studies that revealed fundamental or systems level insights into cellular functions and metabolism.

Category	Major findings	SynBio approach and techniques	Analytical, omics and modeling techniques	Reference
In vivo enzyme reversibility	Pyruvate formate lyase (Pfl) can condense acetyl-CoA and formate to pyruvate *in vivo.*	Approach: Creation of selection strain that cannot grow on acetate (Δ*aceA*). Overexpression of Pfl and cultivation on acetate and formate. Techniques: Gene deletions, heterologous gene expression, growth-coupled design	^{13}C-labeling of proteinogenic amino acids	[29]
	6-phosphogluconate dehydrogenase (Gnd) can carboxylate ribulose 5-phosphate *in vivo*, increased NADPH production increases its reaction rate.	Approach: Creation of selection strain that can only grow when Gnd carboxylates ribulose 5-phosphate. Techniques: Gene deletions, heterologous gene expression, growth-coupled design, ALE	NGS, RT-qPCR, ^{13}C-labeling of proteinogenic amino acids	[30]
In vivo enzyme promiscuity	Low specificity L-threonine aldolase (LtaE) can also condense formaldehyde and glycine to serine. 2-keto-3-deoxy-L-rhamnonate aldolase (RhmA) catalyzes the condensation of pyruvate and formaldehyde to 4-hydroxy-2-oxobutanoate.	Approach: Creation of auxotrophic strains that depend on the promiscuous reaction for growth (either on LtaE or RhmA). Expression of pathway enzymes and growth analysis. Techniques: Gene deletions, heterologous gene expression, growth-coupled design	^{13}C-labeling of proteinogenic amino acids, flux balance analysis	[31]
Underground metabolic pathways	Two novel isoleucine biosynthesis routes in *E. coli*: 1. From homoserine 2. From propionyl-CoA	Approach: Creation of an isoleucine auxotrophic strain. Prolonged cultivation under selective conditions lead to evolution of strains. Techniques: Gene deletions, ALE, recombineering to introduce point mutations	NGS, RT-qPCR, ^{13}C-labeling of proteinogenic amino acids, LC-MS	[38]
	Three novel routes for β-alanine production in *E. coli*: 1. Through uracil degradation 2. Via an evolved ornithine decarboxylase 3. Probably via polyamine degradation	Approach: Creation of β-alanine auxotrophic strains. Expression of a mutator plasmid and cultivation under selective conditions. Detection of novel biosynthesis routes. In-depth analysis of evolved strains. Techniques: Gene deletions, mutator plasmid combined with ALE, introduction of point mutations	NGS, RT-qPCR, gas chromatography (GC)-MS	[39]
	Three novel routes to pyridoxal-5-phosphate in *E. coli*: 1. From serine biosynthesis (characterized in detail) 2. Unknown origin 3. Unknown origin	Approach: Creation of a pyridoxal 5′-phosphate auxotrophic strain. Expression of a library of > 3000 native ORFs in the auxotroph strain and cultivation under selective conditions to identify ORFs that can contribute to pyridoxal 5′-phosphate biosynthesis. Techniques: Gene deletions, native gene overexpression library	Enzyme assays, Analysis of pathway metabolites by ^{1}H NMR	[40]
	A novel route for phosphoglycolate detoxification during autotrophic growth in *C. necator*: 1. The malate cycle for complete oxidation of phosphoglycolate	Approach: Generation of several deletion strains to determine different routes of phosphoglycolate metabolism. Techniques: Gene deletions.	RNA-seq based transcriptomics, Ion chromatography of secreted metabolites	[42]
Gene function and metabolic networks	Determination of essential and growth-supporting genes of *V. natriegens.*	Approach: Pooled genome-wide CRISPRi screen that repressed 1 gene/cell. Pooled growth and determination of gRNA abundance to determine essential genes. Techniques: CRISPRi screen, transposon mutagenesis	NGS, RNA-seq based transcriptomics	[43]
	Metabolic robustness of *E. coli* upon downregulation of several metabolic enzymes via CRISPRi was demonstrated including 3 buffering/by-pass mechanisms.	Approach: Pooled CRISPRi screen that downregulates >1500 target genes. Characterization of metabolome and proteome responses in 30 CRISPRi strains, elucidation of buffering mechanisms Techniques: CRISPRi screen	NGS, RNA-seq based transcriptomics, Proteome analysis, Metabolome analysis, constraint-based stochiometric modeling and kinetic modeling	[44]

Evolved synthetic metabolism	Establishment of a functional CBB in *E. coli* for growth on CO_2 and formate. Functional operation of this autocatalytic cycle was demonstrated to require kinetic mutations in enzymes at branching points that convert CBB-cycle intermediates towards biosynthesis.	Approach: Creation of a selection strain which is dependent on full CBB cycle operation to supply upper metabolisms, which was evolved to hemi-autotrophic growth in a xylose-limited chemostat. Techniques: Gene deletions, heterologous gene expression, growth-coupled design, ALE	NGS, ^{13}C-labeling of proteinogenic amino acids, HPLC analysis of supernatant, constraint-based stochiometric modeling	[8,46]
	Establishment of the reductive glycine pathway in *E. coli* for growth on formate or methanol. Pathway operation could be improved by increased expression of a bottleneck enzymes and improved NADPH generation.	Approach: Creation of a formate-dependent selection strain. Stepwise implementation of pathway modules. ALE of engineered strain to achieve formatotrophic growth. Techniques: Gene deletions, heterologous gene expression, growth-coupled design, ALE	NGS, RT-qPCR, ^{13}C-labeling of proteinogenic amino acids	[9]
	Establishment of the non-oxidative glycolysis (NOG) in *E. coli* by extensive laboratory evolution, including mutations upregulating pathway enzymes and downregulating enzymes draining pathway intermediates.	Approach: Creation of a selection strain that can only metabolize sugars to acetyl-CoA via NOG. ALE of engineered strain in glucose minimal medium with decreasing amounts of acetate. Techniques: Gene deletions, heterologous gene expression, growth-coupled design, ALE	NGS, RT-qPCR, ^{13}C-labeling of acetate and formate, kinetic modeling	[51,52]
	Establishment of the ribulose monophosphate pathway (RuMP) in *E. coli* for growth on methanol. Several genome-wide adaptations were needed, including mutations to balance the levels of the toxic pathway intermediate formaldehyde and to upregulate pathway enzymes.	Approach: Creation of a selection strain that requires methanol assimilation for growth on xylose. A combination of rational mutations and evolution finally allowed for growth on methanol as sole carbon and energy source. Techniques: Gene deletions, heterologous and native gene overexpression, growth-coupled design, ALE	NGS, RT-qPCR, RNA-seq ^{13}C-labeling of formate and acetate, proteomics, kinetic modeling	[49]

it was shown that the *E. coli* enzyme 2-keto-3-deoxy-L-rhamnonate aldolase (RhmA) can catalyze a completely novel reaction i.e. the condensation of pyruvate and formaldehyde to 4-hydroxy-2-oxobutanoate previously shown only *in vitro* by Hernandez et al. [32].

Such promiscuous enzyme activities can contribute to what is called underground metabolism. By definition, underground metabolism describes metabolic conversions that are catalyzed as promiscous reactions of enzymes with endogenous metabolites that do not play a major role under physiological conditions [33]. Underground reactions are thought to contribute to metabolite damage, as "right reactions with wrong substrates" or "wrong reactions with right substrates" can lead to the production of toxic metabolites [34]. On the other hand, it is generally believed that "underground" enzymatic reactions have contributed to the emergence of novel metabolic routes in evolution [35]. As many enzymes act on several substrates (for *E. coli* it has been estimated that 37% of its enzymes act on several substrates and catalyze 65% of the known reactions [36]), underground metabolism presents a fruitful ground for the discovery of novel biocatalysts and could be harnessed for the engineering of novel metabolic pathways [37].

For the discovery of underground reactions and for deciphering the host metabolism gene deletion strains present a great resource. Studying metabolic plasticity in *E. coli*, Cotton et al. showed that underground metabolism can be exploited to replace known amino acid biosynthesis routes [38]. By deleting the canonical isoleucine biosynthesis route, the authors challenged *E. coli* to use underground reactions to compensate for the missing pathway. After a prolonged lag-phase, the deletion strains started to grow, using one of two previously unknown routes for isoleucine biosynthesis, an aerobic route via homoserine or an anaerobic route via propionyl-CoA. In a similar effort, the metabolic plasticity of *E. coli* and the emergence of novel metabolic routes were demonstrated in a β-alanine auxotroph strain [39]. Using a mutator plasmid to enhance mutation rate and serial dilutions under selective conditions, Pontrelli et al. detected first the emergence of a pathway that enabled β-alanine production through uracil degradation. Upon the deletion of this route, a second pathway emerged that uses a mutated enzyme of the ornithine degradation pathway for a novel route towards β-alanine. A different approach was used to identify promiscuous enzyme activity that can restore growth of a pyridoxal-5-phosphate auxotroph strain [40]. Instead of evolving the strain, the authors overexpressed a library of native enzymes and selected for growth in minimal medium. They found that seven enzymes could restore growth via three novel routes that lead to pyridoxal-5-phosphate synthesis. Yet another underground route was recently discovered in the chemolithoautotrophic bacterium *Cupriavidus necator*. Like

other autotrophs that use the Calvin–Benson–Bassham (CBB) cycle, *C. necator* needs to metabolize phosphoglycolate, the oxygenation site-product of ribulose-1,5-bisphosphat-carboxylase/-oxygenase (rubisco) during CO_2 fixation. It was found that the dominant phosphoglycolate salvage route in *C. necator* is the glycerate pathway, also used by cyanobacteria [41]. However, by using gene deletion strains it was revealed that *C. necator* also possesses another metabolic option for phosphoglycolate metabolism, an underground route termed the "malate cycle" that completely oxidizes phosphoglycolate to CO_2 [42].

The previous examples clearly highlight the potential of gene deletion strains for the *in vivo* analysis of enzymatic reactions. However, gene deletions are extreme interventions that do not reflect natural perturbations and cannot be easily applied to essential genes. For studying metabolic responses to temporary or subtle changes in enzyme expression levels, CRISPRi presents a suitable method. It is based on targeted repression of transcription by a nuclease-deficient CRISPR-Cas protein. CRISPRi is becoming a popular tool in functional genomic studies as it enables to deduce gene–phenotype relationships by specific downregulation of gene expression allowing for more precise genome annotations. For instance, a CRISPRi screen has been recently applied to decipher the metabolism and gene functions of the fastest growing bacterium *Vibrio natriegens* [43]. Combining pooled downregulation of all genes with a growth competition assay, the authors were able to classify genes according to their function and essentiality (e.g., only 2 out of 4 predicted enzymes of the L-leucine biosynthesis route were identified as functional). In another recent study, Donati et al. targeted a large number of enzymes in *E. coli* with CRISPRi to address the question of how metabolism reacts when enzyme levels are down-tuned [44]. This fundamental study analyzed 30 selected strains with enzyme targets distributed across metabolism and performed proteome and metabolome analysis on these strains. Hereby, the authors showed that *E. coli* metabolism is robust against substantial decreases of enzyme levels (e.g. by inducing the expression of another bypass pathway) and revealed several "underground" buffering mechanisms (e.g., changes in concentration of allosteric effectors buffered the knockdown of the respective enzyme).

Adaptive laboratory evolution enables synthetic metabolism and provides systems-level insights to metabolism

For the establishment of synthetic metabolism, such as the change of the trophic mode of an organism, rational engineering is often not sufficient, as large-scale rerouting and balancing of metabolism often requires a series of adaptations. Given the high complexity of metabolic systems, it is hard to rationally predict all genetic targets that need to be tackled [45]. In such case, a combination of engineering and ALE can be instrumental to achieve the desired metabolic phenotype. The rationale behind ALE is that cultivation under selective pressure will lead to an accumulation of beneficial mutations that at some point will enable the desired metabolism. After achieving the metabolic goal via ALE, the analysis of the evolved strain can provide novel insights into the host metabolic network and its adaptability, so far a rarely touched source for systems biology studies.

Studies aiming to establish new trophic modes in microbes via synthetic metabolism are commonly aiming to enable assimilation of more sustainable feedstocks for bio-production. A big step in this area is the conversion of a heterotrophic microbe into an autotrophic organism. This was demonstrated some years ago by establishing a functional CBB cycle in *E. coli* [46]. The so-called hemiautotrophic growth (i.e., half-autotrophic, as only the upper part of metabolism was supplied by the CBB cycle) was achieved in an engineered selection strain with separated upper and lower metabolism. The strain was equipped with enzymes required for the CBB cycle that are not present in *E. coli* (rubisco, phosphoribulokinase, and carbonic anhydrase). However, after introduction of these genes, the strain was not able to run the full CBB cycle to supply upper metabolism autotrophically and regenerate ribulose-1,5-bisphosphate required for CO_2 fixation by rubisco and a cyclic pathway activity. To achieve a functional CBB cycle, the strain was subjected to ALE in a continuous evolution system with limiting amounts of xylose (which supplied ribulose-5-phosphate) in the medium. After 50 days, strains had evolved that were capable of growing without xylose. These strains supplied their complete upper metabolism with carbon derived from CO_2 fixation via the CBB cycle. Evolved, hemi-autotrophic strains from three independent evolution experiments had acquired all together about 80 mutations. Comparative analysis and reverse engineering of selected mutations, revealed that only 5 mutations were required to enable CBB cycle activity [47]. Three of these mutations were shown to influence the kinetics of enzymes at branching points of the CBB cycle that drained metabolites out of the cycle for biosynthetic purposes. Supported by kinetic modeling, it was demonstrated that such mutations of branching enzymes are key to enable robust operation of autocatalytic cycles like the CBB cycle [48]. The other two essential mutations involved metabolic regulators of *E. coli* central metabolism. This hallmark study shows how the realization of synthetic metabolism can help to

reveal and understand systems-level principles in metabolism.

In recent years, further progress was made by achieving several engineered strains that can grow on CO_2, methanol or formate as sole carbon source via several synthetic C_1 assimilation pathways. This included the realization of complete autotrophic growth via the CBB cycle in *E. coli* based on the previous work discussed above [8]. Moreover, *E. coli*'s growth on formate and methanol was realized via the reductive glycine pathway [9], and on methanol via the ribulose monophosphate pathway (RuMP) [49]. Synthetic C_1 pathways have also been realized in other organisms: i.e., the CBB cycle in the yeast species *Pichia pastoris* [50] and the reductive glycine pathway in the bacterium *C. necator* [22]. All these studies applied ALE to either realize the novel trophic phenotype or to optimize the growth rates via the synthetic pathway. Some of these studies have already identified essential mutations for the novel metabolic phenotypes. This included for example mutations that were needed to properly (re)balance ratios of redox co-factors such as NADH and NADPH. Also, in several of the studies mutations increasing expression levels of native or heterologous enzymes involved in the synthetic pathways were found. These tuning mutations were often required to realize or improve growth via the synthetic pathway revealing kinetic bottlenecks in the engineered strains.

ALE was also required for the establishment of an alternative version of glycolysis in *E. coli*. The so-called non-oxidative glycolysis (NOG) that avoids carbon loss from pyruvate decarboxylation during acetyl-CoA formation, was designed to achieve a 100% carbon yield from sugars [51]. This synthetic pathway was established by evolving a strain deleted in native pathways for sugar metabolism that relied on NOG for acetyl-CoA production [52]. In the NOG pathway, the flux is diverted from glycolysis to the pentose phosphate pathway where acetyl-CoA is produced via acetyl-phosphate generated by the enzyme phosphoketolase (xylulose-5-P - > glyceraldehyde-3P + acetyl-P; fructose 6-P - > erythrose 4-P + acetyl-P). The evolution of the highly engineered strain was conducted in glucose minimal medium with decreasing amounts of acetate. A plethora of mutations was identified via NGS, most notably several transposon insertions that lead to gene inactivation and downregulation of some overexpressed pathway genes, which probably lead to expression burdens.

While the presented studies had clear engineering targets, they nicely display the power of ALE to also increase our understanding of metabolic networks and their rewiring. Such studies could benefit from performing multi-omics analyses on the evolved strains, including proteomics and metabolomics, as well as integrate these data with stoichiometric and kinetic metabolic models. This would help to further understand systems-level principles and regulatory mechanisms of metabolic networks and the evolution of metabolism [53–55]. Overall, synthetic metabolism studies represent an enormous untapped resource for systems biology.

Conclusion

Synthetic biology approaches have been instrumental in revealing the reversibility and promiscuity of enzymatic reactions in a cellular context, as well as uncovering underground metabolism (see Table 1). We expect that rapidly expanding synthetic biology toolboxes for an increasing range of (non-model) organisms will further support the discovery of "hidden" metabolic reactions in diverse hosts and conditions. For the establishment of synthetic metabolism, in several cases rational engineering was complemented by ALE (see Table 1). By using this combination, some important milestones in synthetic metabolism i.e. establishing *E. coli* growth via the CBB cycle, the RuMP cycle, the NOG pathway, as well as the reductive glycine pathway were recently accomplished. These studies have begun to pave the way to provide optimized enzymes, pathways and strains for a sustainable bio-economy. However, these engineered strains have so far only been analyzed at the level of genomics, occasionally also at a transcriptomic level. This level of strain analysis only provides a shallow insight into the real metabolic situation of the evolved microbe, and can only partially help to deduce underlying biological mechanisms. For a better understanding of the evolved metabolic network, in-depth analysis of the synthetic organism at a multi-omics level, including proteomics, metabolomics and fluxomics can provide deeper insights into the structure of metabolism and provide evidence of the function and evolution of natural and synthetic metabolism.

We believe that in the coming years a stronger integration of synthetic biology into systems biology will be of great benefit to increase our understanding on metabolic systems. To increase this synergy, it is crucial to apply the tools of synthetic biology to design and engineer strains with the specific aim of answering outstanding questions in metabolic systems.

Conflict of interest statement

Nothing declared.

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This milestone article demonstrates the conversion of *E. coli* into an autotrophic organism capable of growing via the CBB cycle. Engineering and chemostat evolution were combined to achieve this goal. Several key mutations in flux branch points and pathway enzymes were discovered.

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E. coli was engineered to grow on formate and methanol via the newly introduced reductive glycine pathway, the first time synthetic formatotrophy was demonstrated *in vivo*. Adaptive laboratory evolution was used to adapt the strain to higher formate concentrations and enable better growth via the pathway. NGS analysis revealed several key mutations.

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