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Engineering carbon fixation in *E. coli*: from heterologous RuBisCO expression to the Calvin-Benson-Bassham cycle

Abstract

Carbon fixation is the gateway of inorganic carbon into biosphere. Our ability to engineer carbon fixation pathways is expected to play a crucial role in the quest towards agricultural and energetic sustainability. Recent successes to introduce non-native carbon fixation pathways into non-photosynthetic hosts offer novel platforms for manipulating these pathways in genetically malleable organisms. Here, we focus on past efforts and future directions for engineering the quantitatively most significant carbon fixation pathway in the biosphere, the Calvin-Benson cycle, into the well-known model organism *Escherichia coli*. We describe how central carbon metabolism of this heterotrophic bacterium can be manipulated to allow directed evolution of carbon fixation enzymes and highlight future directions towards synthetic autotrophy.

Introduction

Carbon dioxide (CO_2) fixation is the most central biological process connecting the inanimate and living world. Out of the six known carbon fixation pathways found in nature [1–3], the Calvin-Benson-Bassham (CBB) cycle (Figure 1a) is the primary carbon assimilation pathway of the biosphere [4], and due to its pivotal role in agricultural productivity, considered the most economically-relevant. Photoautotrophs, such as plants, algae and cyanobacteria fix about 300 gigatons of CO_2 from the atmosphere annually, an amount which exceeds the amount emitted annually by the activities of the global human population by about tenfold [5]. In agriculture, where water and nutrients are abundant, the enzymatic rate of carbon fixation can limit the growth rate of plants, and hence agricultural productivity [6]. For example, various plants have shown a significant increase in growth rate when exposed to an elevated atmospheric CO_2 concentration due to increased carbon fixation rate [7].

An extensive literature covers the biotechnological efforts to improve carbon fixation rate of the CBB cycle [6,8–12]. These attempts mainly focused on the apparent shortcomings of the CO_2-fixing enzyme in the cycle, Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO): a slow catalytic turnover rate and relatively low specificity towards CO_2 that results in an undesired oxygenation side-reaction.

However, despite intensive research and biotechnological efforts, achieving a superior enzyme that could improve photosynthesis in plants is still a standing challenge.

In the last decade, efforts to manipulate carbon fixation have been extended beyond the scope of photosynthetic autotrophs by attempts to genetically engineer model heterotrophs, such as *Escherichia coli* and *Saccharomyces cerevisiae,* to recombinantly express carbon fixation pathways [13–16]. The use of engineered heterotrophs, which do not natively utilize CO_2 as a carbon source, complements the research conducted in autotrophs and offers an extended experimental toolbox to tackle biotechnological challenges towards enhancing CO_2 fixation rates. In this review, we focus on the applications of *E. coli* as a platform to study and manipulate carbon fixation pathways, and specifically the CBB cycle.

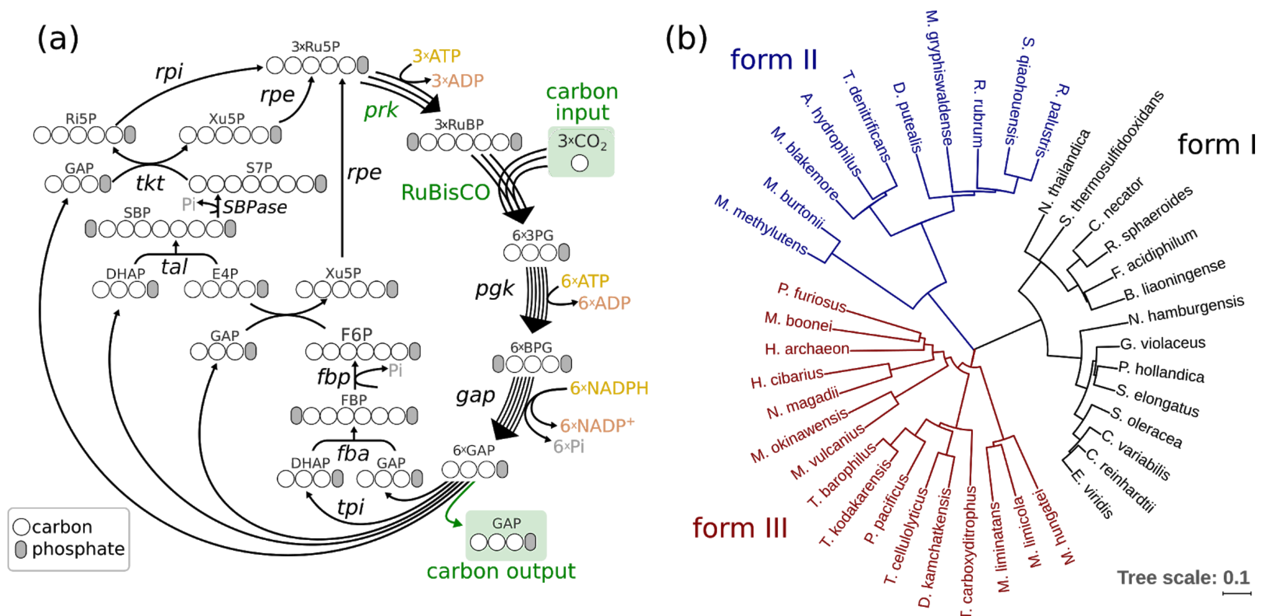

Figure 1: The Calvin–Benson–Bassham (CBB) cycle - the most quantitatively significant pathway for CO_2 fixation. (a) Metabolic diagram depicting the enzymes and intermediates in the CBB pathway. (b) Phylogenetic tree of selected RuBiSCO large subunit sequences. Different RuBiSCO classes are colored: Form I (black), Form II (blue), and the archeal Form III (red). Panel (a) adapted from Berg, J. M., Tymoczko, J. L. & Stryer, L. Biochemistry. (W. H. Freeman, 2007).

Heterologous expression of RuBisCO in *E. coli*

E. coli has long been a preferred host for the manipulation and production of recombinant proteins, and as such, a natural choice for early attempts, during the 1980s, to recombinantly express RuBisCO-encoding genes [17–19]. These pioneering efforts not only demonstrated the ability to manipulate RuBiSCO outside the context of an autotrophic host, but also revealed a surprising interaction between RuBisCO and *E. coli's* native proteins. Unexpectedly, the bacterial "heat-shock" complex (now known as groEL)

was essential for the formation of active RuBisCO [20]. This finding, together with other studies that identified in the chloroplast a ‘RuBisCO binding protein’ as a homolog of bacterial groEL, led to the understanding that auxiliary proteins play a vital role in the biogenesis of RuBisCO (for an excellent recent review see [21]), and contributed to the establishment of the “molecular chaperones” concept [22,23].

While the catalytic mechanism and the structure of the active site of RuBisCO are highly conserved, several structural variants are found across the tree of life [24,25]. The most abundant variant, known as Form I, is a hexadecameric complex found in higher plants, eukaryotic algae, cyanobacteria and proteobacteria. The From I holoenzyme is composed of eight large subunits and eight small subunits. Form II RuBisco is found in different types of proteobacteria and in one group of eukaryotes, the dinoflagellates. It is composed only from large subunits and the holoenzyme is assembled from one or more dimeric pairs. More recently a third RuBisCO clade (Form III) was identified in archaea, in which dimers of the large subunit, as well as higher order assembly (for example, a pentagonal ring of five dimers), were observed. Phylogenetic map from selected sequences of all three forms is shown in Figure 1b.

Generally, the native chaperone network of *E. coli* is sufficient for attaining high yield of active Form II enzymes upon recombinant expression. In contrast, expression of the structurally more complex Form I often fails to produce a catalytically active complex and results in the accumulation of insoluble aggregates. Exceptions to this observation are some prokaryotic Form I RuBisCOs that can be functionally expressed in *E. coli*, but with a relatively low fraction of properly assembled complex [18]. Crucially, Form I RuBisCO of eukaryotic organisms, and most notably of crop plants, fail to assemble catalytically active holoenzyme, indicating that additional factors beyond *E. coli* native chaperones are essential to complete the multi-step biogenesis of the enzyme in this host [19,26,27]. Indeed, it was found that recombinant co-expression of auxiliary proteins from autotrophic organisms, such as RbcX or Raf1, can significantly enhance the properly assembled fraction of some of the Form I RuBisCOs [28,29], yet the functional expression of a plant or algal RuBisCO in a heterotrophic host remains a major challenge for the community.

The ability to express RuBiscO in a genetically malleable organism enabled the construction of synthetic designs using *E. coli* as a host. Examples include fusion peptides, in which the large and small subunits of cyanobacterial RuBisCO were fused using a linker peptide [30]. Another approach aimed to assemble hybrid holoenzymes by co-expressing a cyanobacterial large subunit with a small subunit derived from two eukaryotic marine organisms, *Cylindrotheca sp.* N1 and *Olisthodiscus luteus* [31]. While some of the

designs demonstrated mildly improved specificity for CO_2 *in vitro*, no improved properties of a synthetic RuBisCO have yet been demonstrated *in vivo*.

Directed evolution of RuBisCO activity in an *E. coli* host

The richness of genetic tools and the high transformation efficiency of *E. coli* motivated its utilization as a platform for the directed evolution of RuBisCO towards improved kinetic properties [32]. In a typical directed evolution experiment, a library is constructed by introducing mutations into a known protein sequence, either by design or by random mutagenesis. The library is then transformed into a host that enables to screen the properties of the different variants and the isolation of those with desired properties [33]. While autotrophic organisms offer a direct approach to screen for RuBiSCO activity based on its essentiality for autotrophic growth [34–38], the transformation efficiency and the available genetic tools for these organisms are still severely limited in comparison to those of model heterotrophs such as *E. coli*. However, as heterotrophic organisms are unable to grow solely on CO_2, the development of an appropriate screen was a conceptual and technical challenge.

In photosynthetic organisms, the designated kinase phosphoribulokinase (*prk*) mediates the phosphorylation of ribulose-5-phosphate to yield ribulose-1,5-bisphosphate (RuBP), the substrate of RuBisCO (Figure 1a). However, *E. coli* lacks *prk* as RuBP is not a native intermediate in its metabolic network and recombinant co-expression of this enzyme is therefore required [39]. Upon the expression of *prk,* ribulose-5-phosphate, a central intermediate in the pentose-phosphate pathway of *E. coli*, is readily phosphorylated to yield RuBP *in vivo.* Notably, no native enzyme in *E. coli* consumes RuBP. Therefore, if *prk* is expressed in the absence of a functional RuBisCO, RuBP rapidly accumulates and induces toxic effects that hamper the viability of the host [39].

Parikh et al. took advantage of RuBP-toxicity to implement a synthetic screening system for RuBisCO in *E. coli* , as depicted in Figure 2a. The screen is based on the ability of RuBisCO to metabolize RuBP and prevent the deleterious effect of its accumulation [13]. By controlling *prk* expression using an arabinose inducible promoter (P_{BAD}), the kinase expression can be tuned to saturate the metabolic capacity of RuBisCO and induce toxic RuBP accumulation. Library variants with fitness gain, arising ideally from mutations that improve the kinetics of RuBisCO and enable rapid detoxification, can then be identified and analysed.

While this platform was successfully used to screen a library of cyanobacterial Form I RuBiSCO mutants (*S. elongatus* PCC6301), follow up biochemical characterization of clones showing fitness gain indicated that most of the apparent improvement arose from mutations that promote proper folding and assembly of

RuBisCO, rather than superior kinetic properties [40]. Following studies attempted to minimize the selection towards undesired expression-improved mutants by co-expressing RuBisCO (*S. elongatus* PCC7002) with assembly factors such as rbcX, achieving a ≈5 fold increase in the solubility of the wild-type enzyme in the *E. coli* host [41]. Only after the functional expression of RuBisCO reached a "saturated" level, in which individual point mutations could hardly improve its expression level further, RuBP-toxicity based screen was applied and activity-improved mutants could potentially emerge. Using this activity-directed modified system, a library of randomly mutagenized large and small subunits was screened, and a variant containing two point mutations in the small subunit was found to exhibit improved RuBisCO activity. *In vitro* measurements of the mutated enzyme claimed an 85% improvement in the carboxylation rate with only modest decrease in the affinity towards CO_2 [41]. While the preliminary step of expression optimization was beneficial to reduce the emergence of undesired expression-improved mutants, the selection platform was still susceptible to a high rate of false positives which limited its applicability. Loss-of-function mutations in *prk* eliminate the toxic effect of RuBP and allow false positive colonies to emerge and potentially take over the population.

To overcome these limitations of RuBP-toxicity based screening, an alternative selection platform, achieved through a clever metabolic rewiring was implemented by Mueller-Cajar et al. [14,42]. The RuBisCO-dependent *E. coli* (RDE) selection system, depicted in Figure 2b, is based on a glyceraldehyde-3-phosphate dehydrogenase (*gapA*) knockout strain. As *gapA* is essential for glycolysis, this mutant is unable to utilize glucose as a carbon source. Heterologous expression of *prk* and RuBisCO introduces a two step metabolic shunt that bypasses the glycolytic cutoff and rescue growth by enabling carbon flow towards the TCA cycle for energy and biomass production (Figure 2b). As both RuBisCO and *prk* essential for growth in the *gapA* mutant, this design is significantly more robust to false positives in comparison to RuBP-toxicity based selection.

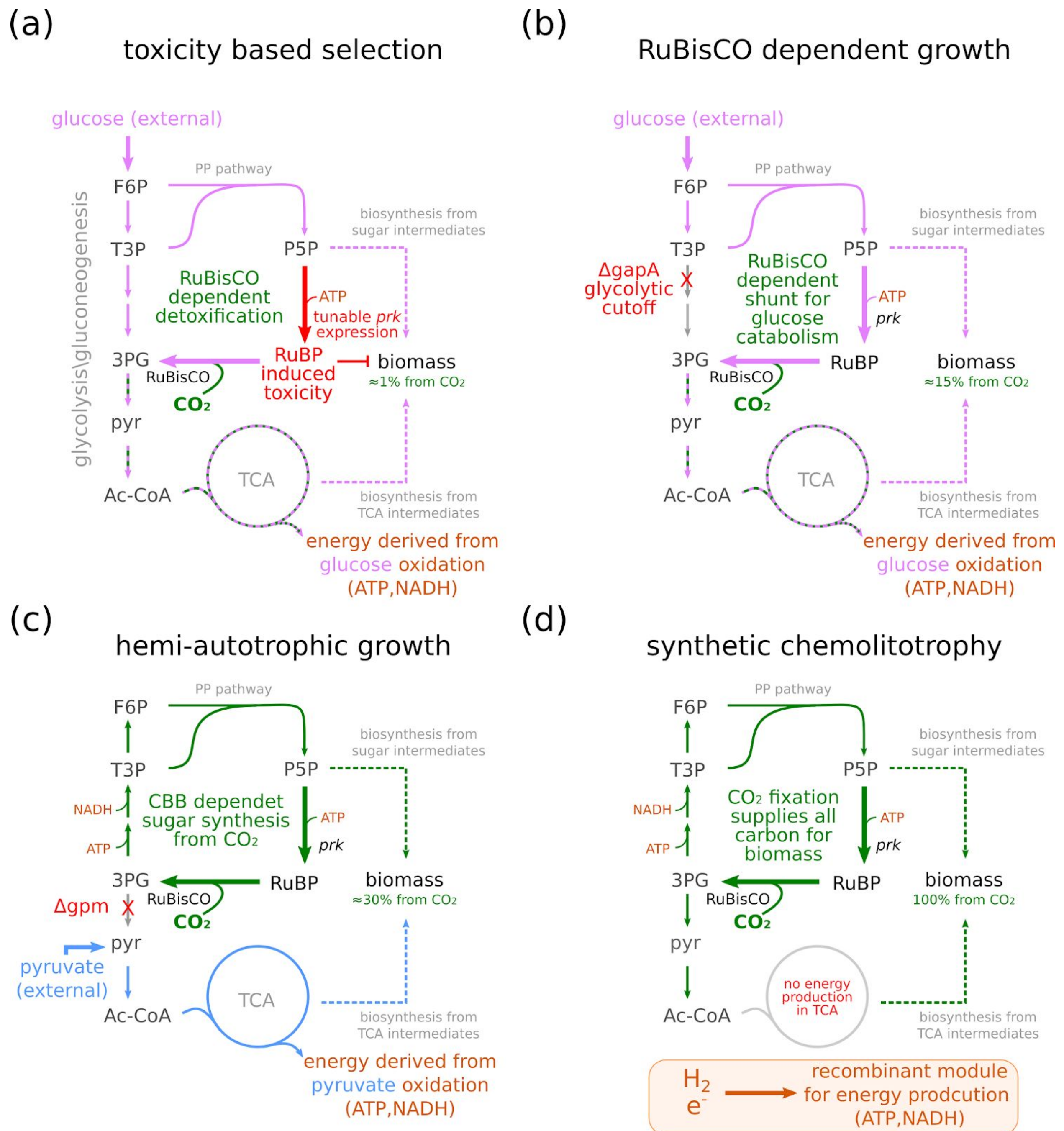

Figure 2: Engineering *E. coli* for carbon fixation. A schematic representation of four metabolic strategies which couple cellular growth to RuBisCO activity is presented. (a) In the absence of RuBisCO, recombinant expression of *prk* diverts carbon flux from the pentose phosphate shunt into a metabolic dead-end, as no native enzyme in *E. coli* consumes RuBP. The accumulation of RuBP is toxic and inhibits growth. Alleviation of RuBP-toxicity is dependent on the activity of RuBisCO which consumed RuBP towards the production of 3PG. (b) Glyceraldehyde 3-phosphate dehydrogenase (*gapA*) knockout strain is unable to grow on glucose as a sole carbon source. Growth rescue is dependent on the functional expression of *prk* and RuBisCO that introduces a two step metabolic shunt that bypasses the glycolytic cutoff and rescue growth by enabling carbon flow towards the TCA cycle for energy and biomass production. (c) Deletion of the phosphoglycerate mutase genes (*gpmA* and *gpmM*) disrupts carbon flow in the glycolytic/gluconeogenic pathway and disconnects the central carbon metabolism into two modules. When pyruvate is supplied, ATP and NADH are produced by the energy module, composed of the reactions of lower glycolysis and the TCA cycle. However, due to the metabolic cutoff, gluconeogenic sugar synthesis can not take occur. The carbon fixation module, composed from the reactions of upper glycolysis, the non-oxidative pentose phosphate pathway, and recombinant *prk* and *RuBisCO*, can utilize the energy for the

synthesis of all phospho-sugars and sugar derived biomass components, composing about 30% of the biomass, from CO_2. (d) To achieve full autotrophic growth in *E. coli* the production of reducing equivalents and ATP from an inorganic source is required. Hydrogen oxidation or the direct electron uptake from an electrode are two potential alternatives for energy harvesting that can be engineered in *E. coli.* In all panels, co-factors such as NADH and ATP are presented only for reactions which consume energy in the CBB cycle.

The RuBisCO-dependent *E. coli* ΔgapA strain was used to evolve a cyanobacterial Form I RuBisCO (*S. elongatus* PCC6301*)* by screening ≈10^5 randomly generated mutants [42]. Several beneficial point mutations were identified, some of which improved the fraction of properly assembled and active RuBiSCO by ≈10 fold, as summarized in [32]. The kinetic properties of the mutated RuBisCOs varied with noticeable reductions in carboxylation rates and ≈30% stronger affinity (K_M) for RuBP [42]. In a different study utilizing this RuBisCO-dependent *E. coli* platform, two rounds of directed evolution led to the identification of a novel point mutation, localized at the interface between the N- and C-terminal domains of the large subunit [43]. This mutation resulted in ≈3 fold increase in the carboxylation rate of RuBisCO, as demonstrated using *in vitro* assays. Moreover, when the evolved RuBisCO sequence was introduced into an autotrophic host (*S. elongatus* PCC6308), a ≈55% improvement in photosynthesis rate was observed, supporting an equivalent wild-type growth rate but with lower investment in RuBisCO protein. More recently, the system was used to evolve an archaeal Form III RuBisCO from *M. burtonii* [44]. Following directed evolution in *E. coli*, evolved archeal RuBisCOs were introduced into *N. tabacum* to measure their effect in a plant model system. While the mutations identified in the the *E. coli* based screen indeed enhanced the performance of the evolved archeal RuBisCOs in comparison to non-evolved sequence, the transgenic plant did not exhibit robust growth: it required elevated levels of CO_2 and accumulated biomass slower than the wild-type plant. Nonetheless, this is a promising indication that variants identified through directed evolution in *E. coli* are relevant for the performances of RuBisCO in plants.

Potential constraints on the directed evolution of RuBisCO

As first hypothesized by Tcherkez et. al. [45,46], the kinetic shortcoming of RuBisCO may stem from difficulty in binding the featureless CO_2 molecule, resulting in a mechanistic tradeoff between CO_2/O_2 specificity and maximum catalytic rate. More recent surveys [47] showed a spread in these kinetic parameters that did not give a tight correlation between specificity and rate, however the dynamic range in those studies was limited and the values are still within the natural variation observed within the tradeoff (or below, but not above, the tradeoff line). Moreover, while directed evolution efforts of RuBisCO gave tantalizing indications of possible improvements, these (for example a 50% improvement in k_{cat} reported by [43]) are still within the spread observed in the experimental trade-off and call for more thorough

analysis of the results. Further research is therefore required to determine whether a trade off that serves as a glass ceiling on kinetic parameters indeed exists.

The small fraction of the natural diversity tested so far shows there is much hidden potential still unexplored. Recent efforts are tapping into previously unaccessible RuBisCO sequences, for example through functional metagenomic from uncultured organisms [37], to further study the kinetics of RuBisCO across the tree of life. One should also note that natural selection might be limited in how far in sequence space it can explore solutions, if RuBisCO is in a local maximum. Rational protein engineering and lab evolution that go beyond random mutagenesis can potentially allow a larger exploration space by several concurrent mutations that will go beyond any "valley of death" for single step protein evolution. Yet such efforts will require potent tools selecting for RuBisCO activity *in vivo*.

The chances of achieving superior RuBisCO variants in a directed evolution setup are dependent on the properties of the screening platform, and especially on the feasible library size and the sensitivity of detection of the desired trait. Despite the significant resilience to false positives in comparison to RuBP-toxicity based system, the RuBisCO-dependent *E. coli* Δ*gapA* strain still suffers from low selection fidelity. Due to the reduced viability of the strain, selection can only be conducted on solid agar plates and colonies require several days to become observable. More powerful screening platforms with increased selection fidelity could potentially drive forward the evolution of RuBisCO towards improved carbon fixation properties. We review such a potent system in the following section.

Engineering a fully functional CBB cycle in *E. coli*

The success in manipulating the metabolic network of *E. coli* to enable the directed evolution of RuBisCO motivated further attempts to introduce a fully functional carbon fixation pathway into this host. Interestingly, out of the dozen reactions utilized in the CBB cycle, RuBisCO and *prk* are the only two enzymes that must be recombinantly introduced to equip the cell with all the enzymatic machinery required for carbon fixation. While the *E. coli* genome does not contain a designated sedoheptulose-bisphosphate phosphatase (in contrast to most photosynthetic organisms utilizing the CBB cycle), RuBP regeneration can be accomplished either by using the native enzymes of the pentose phosphate pathway, in a sedoheptulose-bisphosphate independent manner ,or through promiscuous activity of other phosphatases. However, even if all of the enzymatic machinery required for carbon fixation is expressed, how are reducing power and energy going to be supplied in order to drive the cycle? While autotrophs are able to harvest the required energy from inorganic sources, for example through the

light-capturing photosynthetic machinery, the absence of such energy-harvesting systems is a general challenge for the introduction of complete CO_2 fixation pathways into obligate heterotrophic organisms.

In a recent study, an *E. coli* strain capable of synthesizing all sugar derived biomass components from CO_2 using a non-native CBB cycle was constructed [48]. The design relied on a simple solution to energize the CBB cycle by utilizing the native pathway for energy harvesting in heterotrophs, namely the oxidation of organic carbon in the tricarboxylic acid (TCA) cycle (Figure 2c). To maintain the selection for CO_2 fixation while organic carbon (e.g., pyruvate) is supplied as an energy source, the dissection of the metabolic network into two distinct modules is required. By severing glycolysis through a phosphoglycerate mutase (g*pmA/gpmM*) knockout and the recombinant expression of RuBisCO (*R. rubrum*) and *prk* (*S. elongatus* PCC6301*)*,central carbon metabolism was divided into two metabolic modules: (i) the CO_2 fixation module, composed from the upper glycolysis pathway, the non-oxidative pentose phosphate pathway, RuBisCO and *prk*, (ii) an energy-supplying module, consisting of lower glycolysis and TCA reactions (Figure 3c). As the metabolic cutoff prevents carbon exchange between the two modules, this design reproduces the defining function of the CBB cycle: autocatalytic sugar synthesis from CO_2 with no organic inputs replenishing the cycle intermediates. However, even when ample energy is produced through the TCA (for example, when pyruvate is supplied to the media, Figure 3c), successful integration of the CBB cycle is not naturally occuring due to the complex interplay and interference with existing biosynthetic and energy metabolism. Initially, the re-wired metabolism failed to sustain sugar production from CO_2 and growth was only possible when an external sugar (e.g., xylose) was added to the media [48].

As rational design approaches are often limited in the ability to pinpoint the causes preventing successful integration of a synthetic pathway, adaptive evolution offers an effective alternative to achieve the required fine-tuning. Indeed, a few weeks of laboratory evolution under intense selection in a xylose-limited chemostat were required for the emergence of adapted mutants, in which sugar synthesis from CO_2 was observed. Whole genome sequencing of the evolved strain uncovered that fine-tuning of metabolic enzymes beyond the CBB cycle is essential to enable robust activity of the cycle. Specifically, most of the newly acquired mutations affected enzymes in flux branch points that divert intermediates of the CBB cycle towards biosynthetic routes. Further theoretical analysis [49] suggested that in contrast to linear metabolic pathways, in the case of autocatalytic cycles (such as the CBB cycle) fine-tuning of the kinetic properties of enzymes at branch points is required to sustain a stable metabolic steady-state. Imbalanced kinetics, for example if the affinity of an enzyme at a branch point is excessively strong, can lead to over-draining of intermediates and a metabolic shutdown of the cycle [49].

The CBB cycle-dependent strain offers a novel platform to investigate the biochemistry and metabolic control of carbon fixation, and can be used as a potentially powerful selection system for the directed evolution of RuBisCO in *E. coli*. While the described hemiautotrophic growth is at best neutral in terms of carbon balance, as organic carbon is respired to supply energy and electrons for CO_2 reduction, the modularity of this design suggests a direct path to replace the TCA based energy module with molecular machinery capable of harvesting energy from inorganic sources as we continue to discuss (Figure 1d). The next step to engineer the genetically malleable *E. coli* towards full autotrophy therefore calls for energy supply decoupled from organic carbon oxidation. The ability to radically transform the trophic mode of this model-organism offers an exciting synthetic platform. Synthetic autotrophy in *E. coli* can go beyond what is currently explored in naturally occurring photo- and chemolithoautotrophs, in order to study the constraints and design principles of metabolism, with future potential to serve as a workhorse in carbon fixation applications.We briefly review a few such directions in the following section.

Towards full synthetic autotrophy in *E. coli*

Due to the genetic and structural complexity of the light harvesting machinery, heterologous expression of fully functional photosystem in *E. coli* is still an extreme challenge [50,51]. While recent efforts to construct artificial versions of light harvesting machinery show promising results [52], an alternative solution for energy provision can be obtained from the enzymatic oxidation of reduced chemicals.

Chemolithoautotrophs and methylotrophs employ a wide array of enzymes capable of harvesting reducing power and generating ATP from inorganic sources or reduced one carbon compounds. Such enzymes are significantly simpler and involve fewer components than the multi-gene and structurally complex biological photosynthetic machinery and therefore more suitable to be used as heterologous energy modules. Enzymes capable of oxidizing one carbon compounds such as formate or methanol have been successfully expressed in *E. coli* and can potentially supply the NADH and ATP requirements to drive CO_2 fixation [53,54]. As these compounds are readily obtained from natural gas, or using high efficiency electrocatalysis, they offer relatively cheap, and if produced from electricity derived from a renewable source also potentially sustainable, substrates for the production of sugars and other useful organic molecules from CO_2 [55–57].

In recent years, innovative technologies have enabled highly efficient electrosynthesis of hydrogen by combining photovoltaic solar panels and efficient water-splitting catalysis. Industrially relevant organisms, either natural or synthetic, that could utilize H_2 as an electron source to drive CO_2 fixation offer a promising platform for the renewable production of chemicals [58,59]. Expression of an oxygen

tolerant hydrogenase, capable of oxidizing H_2 while transferring the electrons to the NADH pool, is an attractive option of implementing H_2-based energy module to drive CO_2 fixation in *E. coli*. An additional intriguing alternative which does not rely on soluble electron donors is the direct electron transfer from an electrode [60]. Evidence for direct electron uptake has been demonstrated in microbes, and while the efforts to fully elucidate the molecular details are ongoing, heterologous expression of an electron transfer pathway that enables direct electron transfer in *E. coli* has already been reported [61].

Future prospects

Efforts to introduce non-native one carbon assimilation pathways into heterotrophic hosts span beyond the well-known CBB cycle. Pathways such as the the 3-hydroxypropionate bicycle and the ribulose monophosphate pathway were expressed to various levels of functionality in *E. coli* and their metabolic activities have been demonstrated, though not yet at a level capable of supporting growth in the absence of an organic carbon source [15,54,62]. In addition to natural pathways, heterotrophic model organisms are an attractive platform for testing and constructing synthetic pathways. Several such designs were proposed for achieving synthetic CO_2 fixation as well as in the context of other one carbon molecules such as formate [57,63,64]. Recently, *in vitro* activity was demonstrated for a 17-reaction synthetic CO_2 fixation cycle, composed of enzymes from nine different organisms [65]. Well-studied model organisms such as *E. coli*, for which the metabolic and genetic networks have been extensively studied, are the most amenable platforms for future efforts to assemble such novel CO_2 fixations cycles within the context of a pre-existing metabolic network.

In conclusion, carbon fixation cycles are the metabolic highways that enable the production of organic matter from inorganic carbon building blocks. Our ability to manipulate and improve carbon fixation pathways, and specifically the CBB cycle, has been rapidly moving forward in recent years. Further efforts can help realize their potentially crucial role in facing sustainability challenges like the production of food, biofuels and commodity chemicals in the years to come.

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An especially clear and comprehensive review regarding the role of auxiliary factors in the biogenesis and maintenance of RuBisCO. The cutting edge of understanding regarding the structure and function of chaperones, assembly factors and metabolic repair enzymes is described with specific reference to the impact of RuBisCO engineering efforts.

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The authors develop a robust directed evolution platform for carbon fixation in *E. coli*. By introducing a metabolic cutoff in glycolysis, growth under selective conditions is only rescued upon functional expression of RuBisCO and *prk*. They use this selection system to evolve a cyanobacterial RuBisCO and identify variants with superior properties.

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Directed evolution of an archeal RuBisCO is performed in *E. coli* and promising variants are introduced into *N. tabacum* to assess their performance in a transgenic plant model system. While the evolved RuBisCO did not improve productivity in comparison to wild-type plants, it outperformed the transgenic plant expressing the native archaeal sequence indicating that variants identified through directed evolution in *E. coli* are relevant for RuBisCO performance in plants.

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The evolution of a cyanobacterial RuBisCO in *E. coli* yielded a mutant with catalytically superior properties. When re-introduced into a photosynthetic host, this mutation supported growth at a rate similar to wild-type but with ~25% less investment in Rubisco protein and with a ~55% improved photosynthesis rate per RuBisCO. Furthermore, the authors demonstrate that in contrast to the GroEL/ES promiscuous chaperonin

system that increases the sequence space of functional RuBisCO, a specific assembly chaperone, rbcX, imposes a negative effect on the evolvability.

***[37]** Varaljay VA, Satagopan S, North JA, Witte B, Dourado MN, Anantharaman K, Arbing MA, Hoeft McCann S, Oremland RS, Banfield JF, et al.: **Functional metagenomic selection of ribulose 1, 5-bisphosphate carboxylase/oxygenase from uncultivated bacteria**. *Environ. Microbiol.* 2016, **18**:1187–1199.

A functional metagenomic selection that recovers physiologically active RubisCO molecules directly from uncultivated microbial samples using a RubisCO-deletion strain of *Rhodobacter capsulatus*. This approach provides a new window into the discovery of CO_2-fixing enzymes not previously characterized.

***[63]** Bar-Even A, Noor E, Lewis NE, Milo R: **Design and analysis of synthetic carbon fixation pathways**. *Proc. Natl. Acad. Sci. U. S. A.* 2010, **107**:8889–8894

This study devised an *in silico* methodology for designing and analyzing synthetic carbon-fixation pathways by considering all known enzymatic functions as potential building blocks. The authors present a novel collection of synthetic CO_2 fixation cycles and analyze their kinetic and thermodynamic properties.

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A remarkable *in vitro* implementation of a synthetic carbon fixation pathway with 17 enzymes from nine organisms. This rationally designed pathway utilizes an enoyl–CoA carboxylases/reductases to produce glyoxylate from CO_2 that can potentially exhibit superior kinetics over natural carbon fixation pathways.

***[52]** Sakimoto KK, Wong AB, Yang P: **Self-photosensitization of nonphotosynthetic bacteria for solar-to-chemical production**. *Science* 2016, **351**:74–77.

This study presents a synthetic light-harvesting approach based on cadmium sulfate nanoparticles that can be integrated into living organism. The authors demonstrate a hybrid system in which biologically precipitated nanoparticles can use light to provide the requisite energy for acetate production from CO_2 in a non-photosynthetic host.

***[12]** Lin MT, Occhialini A, Andralojc PJ, Parry MAJ, Hanson MR: **A faster Rubisco with potential to increase photosynthesis in crops**. *Nature* 2014, **513**:547–550.

This study demonstrate functional heterologous expression of cyanobacterial RuBisCO along with a carboxysomal protein in a tobacco plant. The engineered transgenic lines were photosynthetically competent, supporting autotrophic growth, and had higher rates of CO_2 fixation per unit of enzyme than the wild-type tobacco control.

***[51]** Claassens NJ, Sousa DZ, Dos Santos VAPM, de Vos WM, van der Oost J: **Harnessing the power of microbial autotrophy**. *Nat. Rev. Microbiol.* 2016, **14**:692–706.

An impressive overview of recent advances in harnessings CO_2-fixing microorganism toward the sustainable production of chemicals and biofuels. The authors present key engineering strategies to increase productivity in natural autotrophs, as well as in genetically modified CO_2 fixing heterotrophs.

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