Synthetic Carbon Fixation: Conversion of Heterotrophs into Autotrophs by Calvin-Benson-Bassham Cycle Induction

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Summary Synthetic biology has enabled us to artificially convert heterotrophs into autotrophs. Modification of the existing metabolic circuit and induction of enzymes of the Calvin–Benson–Bassham (CBB) cycle including phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) allowed *Escherichia coli* and *Pichia pastoris* to fix CO₂. This heterotroph to autotroph conversion technology will contribute to reductions in energy consumption and CO₂ emissions in the bioindustry.

Keywords Autotrophs, Heterologous, CBB cycle, RuBisCO, PRK, Synthetic biology.

Autotrophs can produce organic compounds from inorganic compounds by carbon fixation. Heterotrophs, however, cannot produce carbon from inorganic compounds, so they must eat organic compounds produced by autotrophs to live. Humans, as consumers of large amounts of fuel and food, are also directly or indirectly dependent on autotrophs. Since the industrial revolution, the burning of fossil fuels and deforestation have caused a significant increase in the concentration of CO₂, one of the greenhouse gases, which has become a major social problem (Kumar *et al.* 2018, Irfan *et al.* 2019, Hosokawa and Kawano 2020). Originally, the balance of the CO₂ concentration in the atmosphere was maintained through CO₂ fixation by autotrophs, but our consumption activities have caused excessive CO₂ emissions. To achieve a sustainable society, we need to minimize CO₂ emissions and increase CO₂ fixation (Ort *et al.* 2015). One important approach is to better understand the principles of carbon fixation in autotrophs (Smith and Stitt 2007) and how to enhance it (Kromdijk *et al.* 2016, Schwander *et al.* 2016, South *et al.* 2019). Building synthetic autotrophs is a good way to understand autotrophs because we can learn the factors required for metabolic pathways and the various constraints on natural autotrophs. Currently, heterotrophic organisms are mainly used in biological industrial production because autotrophs are unsuitable due to their slow growth rate and

reaction efficiency; however, if autotrophs become better understood and these factors are improved, industrial heterotrophs could be replaced by autotrophs. This will help to solve social problems, including CO₂ emissions. Thus, researchers in various fields such as cell engineering, molecular biology, and synthetic biology have been working towards this goal.

There are six known carbon fixation pathways in autotrophs (Berg 2011, Bar-Even *et al.* 2012). In particular, the Calvin–Benson–Bassham (CBB) cycle is important because it is widely distributed in most autotrophs, including algae, cyanobacteria, and plants (Liang *et al.* 2020). The cycle has three phases—carboxylation, a reduction reaction, and ribulose 1,5-bisphosphate (RuBP) regeneration—and is composed of 13 enzymes (Bassham *et al.* 1954). A key enzyme in the CBB cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), fixes more than 10 tons of CO₂ from the atmosphere per year (Field *et al.* 1998, Hayer-Hartl and Hartk 2020). RuBisCO is known to have four forms, of which forms I, II, and III are enzymes that catalyze RuBP-dependent carboxylation (Tabita 1999, Tabita *et al.* 2007). Eukaryotic and prokaryotic RuBisCO enzymes are commonly grouped into form I or form II (Whitney *et al.* 2011). RuBisCO is a very inefficient enzyme, with a very slow carboxylation turnover rate of 1–10 reactions per second and a low affinity for CO₂ (Tcherkez *et al.* 2006). This means large amounts of RuBisCO are required to maintain the balance of photosynthesis *versus* metabolism, making RuBisCO the

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most abundant enzyme on earth (Ellis 1979, Feller *et al.* 2008). In addition, competition between carboxylation and oxygenation is rate-limiting, and the byproduct 2-phosphoglycolate is toxic to cells and must be metabolized in the photorespiratory cycle (Hagemann and Bauwe 2016). Therefore, many researchers have attempted to improve the carboxylation rate and CO₂ affinity by molecular modification and protein-directed evolution, but unfortunately, few improvements have been achieved so far (Ducat and Silver 2012, Lin *et al.* 2014, Kubis and Bar-Even 2019). There are several reasons for this lack of progress, such as the complexity of the RuBisCO protein structure, the obscurity of the genetic background of autotrophs, and the absence of genetic engineering tools. Therefore, some researchers have attempted to artificially design not only RuBisCO but also the CBB cycle (Liang *et al.* 2020). The restructuring of RuBisCO and the rewiring of metabolic pathways in heterotrophs is a critical path to understanding autotrophs and achieving sustainability.

There are several issues involved in converting heterotrophs into autotrophs. First, chaperones and auxiliary factors are required for the folding, assembly, and functional maintenance of RuBisCO, and we still need to identify them (Bracher *et al.* 2017, Hayer-Hartl and Hartl 2020). Form I RuBisCO is difficult to construct in heterologous organisms because it requires several different chaperones and cofactors, but form II RuBisCO can be constructed in *E. coli* with only GroEL, a homolog of chloroplast chaperonin Cpn60, and its cofactor, GroES (Aigner *et al.* 2017, Hayer-Hartl 2017). Second, the constructed CBB cycle needs to be connected to the central carbon metabolism pathway so that all biomass is produced using carbon fixation. In autotrophs, glycerate 3-phosphate generated by RuBisCO is regenerated into ribulose 1,5-bisphosphate in the CBB cycle and simultaneously assimilated into the host to sustain life. Third, in artificial autotrophs, reducing power is deficient due to the absence of photosystems such as chloroplasts. Therefore, approaches using non-chemical energy or carbon sources that are not assimilated by the host are required. Finally, the metabolic kinetics at the branching point between the CBB cycle and the glycolytic pathway need to be optimized to maintain a stable metabolism *in vivo*. The optimal model of the carbon metabolism network is simulated when the CBB cycle, the glycolytic pathway, and the tricarboxylic acid cycle are connected in *E. coli* (Barenholz *et al.* 2017).

Previously, attempts have been made to convert heterotrophic model organisms, such as *Escherichia coli*, *Methylobacterium extorquens*, *Saccharomyces cerevisiae*, and *Pichia pastoris*, into autotrophs using the CBB cycle. Here, we introduce these attempts in each species. *E. coli* is the most widely used host organism in all biotechnology industries (Marisch *et al.* 2013). To select RuBisCO with higher activity in *E. coli*, strategies

to express CBB-related genes and screening tools for directed evolution were developed (Parikh *et al.* 2006, Mueller-Cajar *et al.* 2007, Mueller-Cajar and Whitney 2008, Cai *et al.* 2014, Wilson *et al.* 2016, Zhou and Whitney 2019). When genes for RuBisCO and phosphoribulokinase (PrkA) were overexpressed in *E. coli*, CO₂ emissions were reduced (Zhuang and Li 2013). This research showed that wild-type *E. coli* emits 0.731 mol of CO₂ per mol of arabinose consumed, while engineered *E. coli* emits only 0.621 mol of CO₂ per mol of arabinose. This represents a 15% reduction in CO₂ emissions. In addition, Adaptive Laboratory Evolution (ALE) was developed, which uses chemostats to limit the organic carbon source and perform gradual selection (Sonderegger and Sauer 2003, Blount *et al.* 2012). Consequently, hemiautotrophic growth of *E. coli* was enabled using ALE (Antonovsky *et al.* 2016). In that study, to establish the CBB cycle, form II RuBisCO from *Rhodospirillum rubrum* and prkA from *Synechococcus elongatus* were expressed and evolved in xylose-limited chemostats. The result was an *E. coli* strain that produced a third of its biomass from CO₂ and required pyruvate for reducing power and as an energy source for stable growth. In a subsequent study, a strain of *E. coli* that was a fully autotrophic was established by expressing formate dehydrogenase (FDH) from methylotrophic bacteria, knocking out several genes in the glycolytic system, and evolving *E. coli* with chemostats (Gleizer *et al.* 2019) (Fig. 1). Formate is oxidized by FDH, which is the reducing force of the CBB cycle. Sequencing of these *E. coli* clones revealed mutations in genes involved in the branching of the CBB cycle or in the promoter region that controls NADH production, which suggested that a proper balance is required to maintain stable metabolism.

Yeast is commonly used in the large-scale production of bioethanol as an alternative to fossil fuels because of its strong ethanol tolerance (Alper *et al.* 2006, Della-Bianca *et al.* 2013, Nielsen *et al.* 2013). It is also an important host for the industrial production of biopharmaceuticals and useful proteins (Li *et al.* 2015, Xue *et al.* 2017, Duan *et al.* 2018). In the yeast *S. cerevisiae*, RuBisCO and PRK from various origins were investigated and introduced to reduce CO₂ emissions during bioethanol fermentation (Guadalupe-Medina *et al.* 2013, Xia *et al.* 2017, Papapetridis *et al.* 2018). As a result, the CBB cycle, which reoxidizes NADH using CO₂ as an electron acceptor, showed improved ethanol yield, decreased byproducts, and reduced CO₂ emissions. Notably, form I was demonstrated to have higher carboxylation activity than form II when form I RuBisCO from *Ralstonia eutropha* and form II RuBisCO from *Thiobacillus denitrificans* were each heterologously expressed in *S. cerevisiae* (Li *et al.* 2017). In addition, an engineered strain of *M. extorquens* AM1 was designed by implementing the CBB cycle and knocking out the host genes, but this strain was unable to grow continuously using

Fig. 1. Metabolic models of autotrophs living on CO_2 as a sole carbon source. The engineered bacterium (*Escherichia coli*) obtained a biomass production pathway through carbon fixation in the CBB cycle and an energy production pathway through oxidation of formate. These metabolic pathways were optimized using chemostats. The engineered yeast (*Pichia pastoris*) acquired a peroxisome-localized CBB cycle and energy production pathway through methanol assimilation. Sea slugs (*Plakobranthus ocellatus*) in nature can sequester functional chloroplasts from algae into their cells via kleptoplasty. This enables them to produce biomass through the photosystems and the CBB cycle in chloroplasts.

CO_2 as a sole carbon source (Schada von Borzyskowski *et al.* 2018). More recently, PRK and RuBisCO were introduced into *P. pastoris* and two genes of dihydroxyacetone synthase and a gene of alcohol oxidase in the assimilation pathway were knocked out, resulting in the establishment of a fully autotrophic strain that can grow continuously with CO_2 as a sole carbon source and methanol as an energy source (Gassler *et al.* 2020) (Fig. 1). Surprisingly, the growth rate of this strain was improved to the same level as the wild type using chemostats. This suggested that this strain has the potential to become a basic organism for industrial production and contribute to the reduction of CO_2 emissions.

Finally, although there are no reports of converting animal cultured cells into autotrophs, we introduce some reports that may support the idea. In nature, a variety of photosynthetic animals evolved through secondary symbiosis with algae (Rumpho *et al.* 2011, Van Steenkiste *et al.* 2019). In particular, sea slugs can sequester functional chloroplasts from algae into their cells via a symbiotic phenomenon called kleptoplasty, which allows them to live solely on photosynthetic products like autotrophs (Yamamoto *et al.* 2013, Laetz and Wägele 2017, Aoki and Matsunaga 2021) (Fig. 1). For a long time, researchers have discussed whether horizontal transfer of algal genes may have occurred in sea slugs to maintain chloroplasts without algal nuclei during evolution, but

surprisingly, a recent study showed that horizontal transfer has not occurred in the kleptoplastic sea slug *Plakobranthus ocellatus* (Maeda *et al.* 2021). This implies that complex biological machinery such as chloroplasts can be transferred across species; in other words, an animal can also acquire the ability to photosynthesize.

These achievements in synthetic carbon fixation will accelerate the construction of artificial photosynthetic animal cells (Matsunaga 2018, Puri *et al.* 2021). Although there are still open issues to address in the conversion of heterotrophs into autotrophs, further development will reveal universal principles for conversion. Conversion technology based on these principles will contribute to finding an effective solution to reduce humanity's huge energy consumption and carbon dioxide emissions, leading to global environmental improvement.

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