

Review

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Biochemical unity revisited: microbial central carbon metabolism holds new discoveries, multi-tasking pathways, and redundancies with a reason

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Abstract: For a long time, our understanding of metabolism has been dominated by the idea of biochemical unity, i.e., that the central reaction sequences in metabolism are universally conserved between all forms of life. However, biochemical research in the last decades has revealed a surprising diversity in the central carbon metabolism of different microorganisms. Here, we will embrace this biochemical diversity and explain how genetic redundancy and functional degeneracy cause the diversity observed in central metabolic pathways, such as glycolysis, autotrophic CO_2 fixation, and acetyl-CoA assimilation. We conclude that this diversity is not the exception, but rather the standard in microbiology.

Keywords: carbon dioxide fixation; functional degeneracy; genetic redundancy; glycolysis; metabolic pathways; microbial biochemistry.

Introduction: biochemical unity and microbial diversity

“Anything found to be true of *Escherichia coli* must also be true of elephants”, a famous quote from Jacques Monod

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about *biochemical unity*, traces back to the Dutch microbiologist Albert Jan Kluyver. Almost one hundred years ago, Kluyver was among the first to propose that the core of metabolic transformations is conserved between all life forms, most visibly manifested in the canon of glycolysis, tricarboxylic acid (TCA) cycle, and coenzyme A. While the concept of *biochemical unity* is certainly true for all higher eukaryotes and has been instrumental in developing biochemistry as a discipline, its narrow interpretation by many generations of scientists has for a long time blurred our view onto the vast biochemical diversity of microorganisms.

Historically, once a pathway for a given metabolic trait was discovered [e.g., the Embden-Meyerhof-Parnas pathway (EMPP) for glucose degradation, the Calvin-Benson-Bassham (CBB) cycle for CO_2 fixation, etc.], it was often assumed that this route is universal to all other (micro)organisms. However, today we know that there are (several) variations of and even alternative pathways to the EMPP, as well as the CBB cycle, and we have also learned that the TCA cycle does not only function in the oxidation of acetyl-CoA, but can be operated in the reverse direction to fix CO_2 . These discoveries have been fueled by the advent of genome sequencing, which has unraveled the genetic basis of the underlying biochemical diversity.

Why do we observe this apparent biochemical diversity in the central carbon metabolism of microorganisms? In many cases the diversity reflects the physiological adaption of an organism towards different environmental conditions and ecological niches. This diversity can be enabled by *genetic redundancy* (i.e., paralogs of a gene encoding enzymes with distinct functions) or *functional degeneracy* (i.e., structurally and evolutionarily distinct metabolic pathways conferring the same function). Here, we will (1) review several examples of *genetic redundancy* and *functional degeneracy* in central carbon metabolism, (2) highlight the fact that several functionally degenerate pathways can operate in the same organism in parallel,

and (3) explain how different functionally degenerate pathways can serve multiple purposes in parallel, some of which were discovered only very recently, which further adds to the ever increasing metabolic diversity of microbial metabolism.

Genetic redundancy and functional degeneracy underlie the diversity in microbial autotrophy

After its discovery in the 1950s, it was long thought that the CBB cycle is the only autotrophic CO_2 fixation pathway in nature (Bassham et al. 1954). However, since then, genetic redundancy as well as functional degeneracy have been described in microbial autotrophy. The discovery of at least six different CO_2 fixation pathways demonstrates the impressive evolutionary and biochemical diversity of microbial autotrophic CO_2 fixation. It has been argued that this functional degeneracy is the result of specific adaption of a respective microorganism to its ecological niche (e.g., aerobic vs. anaerobic, energy-limited chemotrophs vs. energy-rich phototrophs) (Berg 2011).

The prime example for genetic redundancy in the CBB cycle concerns the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). RubisCO is the key enzyme of the CBB cycle that catalyzes the actual CO_2 fixation step. However, at the same time RubisCO is also known to accept oxygen instead of CO_2 at the active site causing the phenomenon of photorespiration, which strongly limits the efficiency of the CBB cycle. Several theoretical and experimental studies suggest that RubisCO is trapped in a trade-off between CO_2 fixation activity (“speed”) and specificity for the resolving electrophile (CO_2 or O_2 , “specificity”). This trade-off between speed and specificity is apparently tuned to a given optimum, depending on the environmental conditions (Savir et al. 2010; Tcherkez et al. 2006). In bacteria inhabiting environments of fluctuating CO_2 and O_2 concentrations, several RubisCO forms have evolved that co-exist in the same organisms and cover different kinetic extremes from RubisCO type I (slow and specific) to type II RubisCOs (fast and unspecific). These different RubisCOs are expressed according to the local oxygen concentration, which allows the organism to adapt to different environmental conditions, showcasing how genetic redundancy can confer expansion of the ecological niche (Badger and Bek 2008). While for a long time, only RubisCO type I and II were known to be active in the CBB cycle, a type III RubisCO-dependent transaldolase variant of the CBB cycle

has been recently postulated in *Thermodesulfobium acidophilum*, expanding the examples of genetic redundancy in the CBB cycle (Frolov et al. 2019).

Genetic redundancy is also found in the reverse tricarboxylic acid (rTCA) cycle, a functionally redundant pathway to the CBB cycle (Buchanan and Arnon 1990; Evans et al. 1966). This pathway relies on ferredoxin-independent carboxylases, which typically are oxygen-sensitive and thus limit the distribution of the rTCA cycle to anaerobic habitats. *Hydrogenobacter thermophilus* TK-6, however, encodes two isoforms of the oxoglutarate:ferredoxin oxidoreductase; one of which is expressed in anaerobic conditions whereas the other is able to support growth under (micro)aerobic conditions (Yamamoto et al. 2006), again demonstrating how genetic redundancy can allow ecological niche expansion.

While above two examples illustrate how lifestyle flexibility can be achieved by expressing different enzyme isoforms, expansion of the ecological niche can also be realized by maintaining two functionally degenerate pathways in one organism. The gammaproteobacterial endosymbiont of the deep-sea tube worm *Riftia pachyptila* uses this strategy. *R. pachyptila* resides at hydrothermal vents where it is exposed to continuous and fast-changing fluctuations regarding sulfide and oxygen concentrations (Johnson et al. 1988), which are likely translated to its endosymbionts. It is therefore essential for the endosymbiont to adapt to the different conditions by either using the CBB or the rTCA cycle for autotrophic CO_2 assimilation (Markert et al. 2007). Under anaerobic and energy-limiting conditions, the energetically more efficient rTCA cycle is induced in the symbiont, while in the presence of ample energy and oxygen, the symbiont operates the more costly CBB cycle. Note that this variant of the classical CBB cycle is likely more energy efficient due to a pyrophosphate dependent fructose-1,6-bisphosphatase (FBPase) replacing the canonical FBPase one of the standard CBB cycle (Kleiner et al. 2012). The co-occurrence of the genes for these two functionally degenerate pathways was also confirmed in several other symbionts and even a free-living bacterium, *Thiوفlavicoccus mobilis*, recently (Rubin-Blum et al. 2019). The fact that *T. mobilis* can be cultivated opens up the exciting possibility to investigate the activities and interplay of these two degenerate pathways in their host under different conditions. Studying the potential functional degeneracy in autotrophic CO_2 fixation in this microorganism is also of interest from an evolutionary point of view. Does the situation in *T. mobilis* reflect an evolutionary snapshot or are both pathways stably maintained? For deep-sea mussel epibionts, it was hypothesized that a newly-acquired CBB cycle replaced the pre-existing rTCA cycle during evolution (Assié

et al. 2020). Interestingly, the fully functional CBB cycle is assembled from genes of different evolutionary origins presumably by several events of horizontal gene transfer (HGT). The apparent lack of certain rTCA cycle genes, however, needs to be interpreted with a note of caution due to incomplete genome assembly.

While the rTCA cycle requires enzymes distinct from the standard oxidative TCA cycle, most notably an ATP-citrate lyase, it was shown lately that the oxidative TCA cycle can also be reverted under special physiological conditions. The so-called roTCA (for reverse oxidative TCA) cycle is based on the reverse reaction of citrate synthase, which was previously regarded as impossible *in vivo*. However, very high activities of citrate synthase and high CoA/acetyl-CoA ratios make the reaction of citrate synthase reversible, as recently demonstrated in *Desulfurella acetivorans* (Mall et al. 2018). Similarly, a bidirectional TCA cycle depending on the citrate synthase has been described in a *Thermosulfidobacter takaii* strain (Nunoura et al. 2018). Since many autotrophic microorganisms encode the canonical TCA cycle, the surprising discovery that this pathway can be reversed to fix CO₂ might point to a so far overlooked functional degeneracy in microbial autotrophy, which requires more studies on the environmental relevance of the roTCA.

Taken together, evolution has created several ways to establish metabolic flexibility in microbial autotrophy (Figure 1). Both genetic redundancy (i.e., carboxylase isoforms with different catalytic properties) and functional degeneracy (i.e., different CO₂ fixing pathways in one organism) eventually allow to expand the ecological niche of an organism. While this metabolic flexibility arguably is of advantage to increase the overall fitness of an organism (especially in changing environmental conditions), it is sometimes given up for a short-term advantage or the occupation of a novel niche with the concomitant loss of the previous ecological niche (Lee and Marx 2012).

Functional degeneracy and multi-functionality: the 3-hydroxypropionate bi-cycle parts into several metabolic modules

The autotrophic CO₂-fixing 3-hydroxypropionate (3HP) bi-cycle in the green non-sulfur bacterium *Chloroflexus aurantiacus* was elucidated stepwise (Figure 2). Initially, the glyoxylate-generating cycle was described (Strauss and Fuchs 1993), which was later recognized as part of the bi-cycle (Herter et al. 2002). The missing enzymatic steps were

identified subsequently (Alber and Fuchs 2002; Zarzycki et al. 2009). The pathway requires seven ATP equivalents to produce one molecule of pyruvate from CO₂, which is comparable to the energy requirements of the CBB cycle (Bar-Even et al. 2012). However, when including photo-respiration through RubisCO, the 3HP bi-cycle has a higher energetic efficiency than the CBB cycle in its facultative aerobic host organisms.

First discovered in the context of autotrophic CO₂ fixation, the 3HP bi-cycle was very soon recognized for its ability to also serve in organic carbon assimilation (Zarzycki and Fuchs 2011). Several pathway intermediates like acetate, propionate, 3HP and glycolate (via glyoxylate) were shown to be assimilated by *C. aurantiacus* directly via the 3HP bi-cycle, when fed to autotrophically grown cells, indicating that this pathway allows the co-assimilation of all these substrates naturally. Notably, the same organism additionally encodes the enzymes of the glyoxylate cycle, a canonical pathway for acetyl-CoA assimilation. This functional degeneracy in respect to acetate and glycolate/glyoxylate assimilation might infer additional metabolic flexibility of *C. aurantiacus* under certain conditions. This is in line with the fact that when grown aerobically on acetate only the glyoxylate cycle is induced in *C. aurantiacus*, while under anaerobic conditions, the complete 3HP bi-cycle is active (Zarzycki and Fuchs 2011).

While both cycles of the 3HP bi-cycle operate together in *C. aurantiacus*, they might function individually in other bacteria. The genes for the key enzymes of the 3HP bi-cycle, malonyl-CoA reductase (Mcr) and propionyl-CoA synthase (Pcs), are distributed far beyond the phylum of Chloroflexi. Several aerobic anoxygenic phototrophic (AAP) bacteria contain all six *Chloroflexus*-like genes of the first half of the 3HP bi-cycle for the conversion of acetyl-CoA into succinyl-CoA (Zarzycki and Fuchs 2011). Because these organisms are obligate heterotrophs, it is tempting to speculate that this partial first cycle of the 3HP bi-cycle allows those AAPs to convert acetyl-CoA into succinyl-CoA via co-assimilation of two additional bicarbonate molecules, fixed through acetyl-CoA and propionyl-CoA carboxylase, respectively (Figure 2b). Theoretically, one enzyme in this partial pathway could even be forgone. Pcs is a complex three-domain fusion protein that catalyzes the three-step reaction sequence from 3HP to propionyl-CoA within a closed reaction chamber (Bernhardsgrütter et al. 2018). A recent study showed that the reductase domain of Pcs shows a latent carboxylation activity and can be further converted into a reductive carboxylase by only two point mutations (Bernhardsgrütter et al. 2019). However, the resulting drop in catalytic efficiency might have prevented such a variant to arise during evolution so far. Nevertheless, a

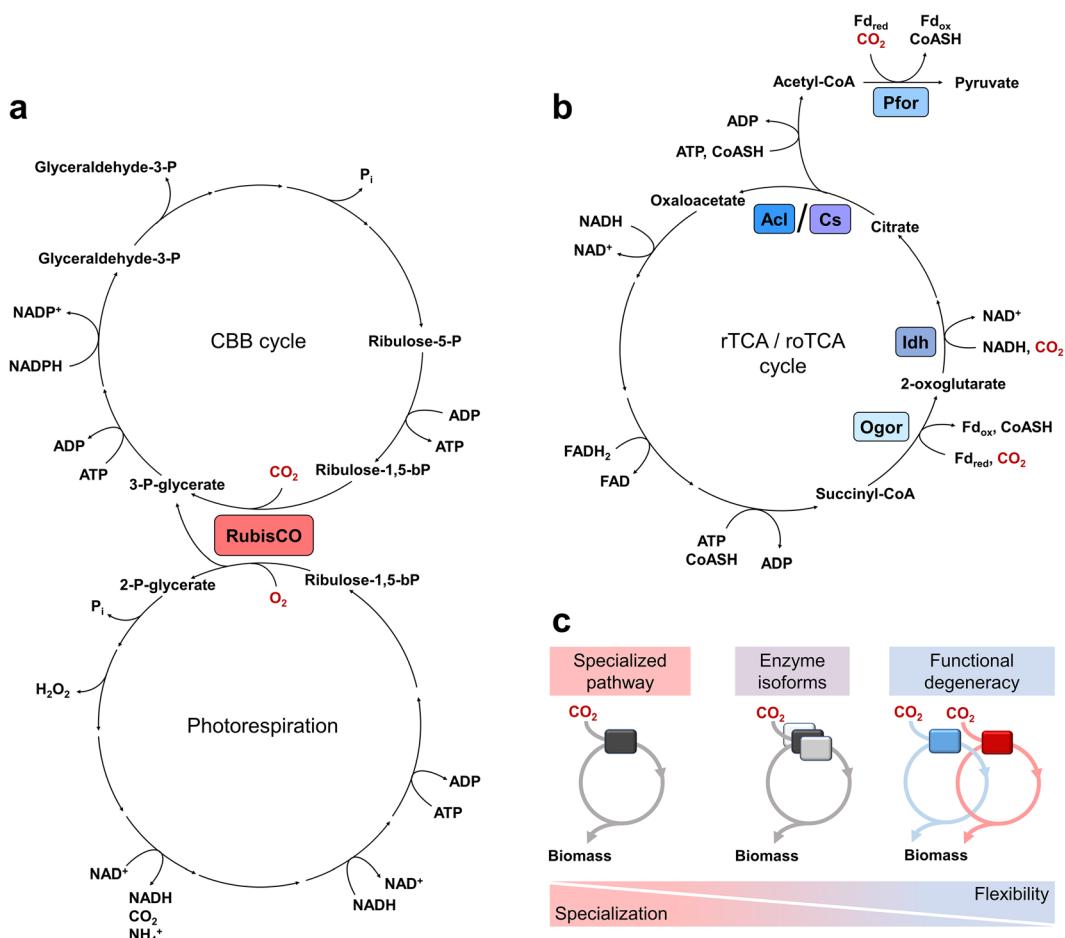


Figure 1: Functional degeneracy and genetic redundancy in microbial autotrophy. (a) Simplified schematic of the CBB and the photorespiration cycle. Isoforms of RubisCO with a different CO₂/O₂ specificity factor determine the flux through either cycle. (b) Simplified schematic of the rTCA or roTCA cycle. In the rTCA cycle the ATP-dependent citrate lyase (Acl) generates acetyl-CoA, a reaction that could also be catalyzed by the citrate synthase (Cs) in the roTCA cycle, as recently shown. These cycles comprise three different carboxylases, the oxoglutarate:ferredoxin oxidoreductase (Ogor), the isocitrate dehydrogenase (Idh) and the pyruvate:ferredoxin oxidoreductase (Pfor). (c) The trade-off between specialization and flexibility of metabolic pathways, illustrated on the microbial autotrophy as an example. Microorganisms can expand their ecological flexibility by expressing enzyme isoforms with different physical or kinetic properties (e.g., RubisCO isoforms with different CO₂ specificity or oxoglutarate:ferredoxin oxidoreductase isoforms with different oxygen sensitivity) or by operating two or more functionally degenerate pathways (e.g., a *Riftia pachyptila* endosymbionts that operate the CBB or rTCA cycle for autotrophic CO₂ fixation). Microbial metabolism is evolutionarily shaped along this trade-off between specialization and flexibility depending on the prevailing environmental conditions and the resulting selective pressures.

carboxylating Pcs could directly yield methylmalonyl-CoA and thereby skip the need for the second, subsequent ATP-dependent carboxylation reaction of propionyl-CoA carboxylase. This acetyl-CoA assimilation pathway would be well-suited to assimilate a variety of organic compounds that AAP bacteria encounter in aquatic habitats under additional co-fixation of CO₂.

Another feature that is common to all AAP bacteria is their ability to utilize light for additional energy conservation. The rudimentary 3HP bi-cycle in AAP bacteria

might be also connected to this process. Photosynthesis causes a surplus of energy and reducing equivalents and thus an imbalance in the redox homeostasis. In anaerobic organisms, it has been shown that reductive metabolic pathways like the CBB cycle or some rTCA cycle enzymes are important to maintain redox homeostasis during photosynthesis (McCully et al. 2020). Despite the obligately aerobic lifestyle of AAP bacteria, several studies have reported a reduced respiration rate in light, which suggests that reductive metabolic pathways in these

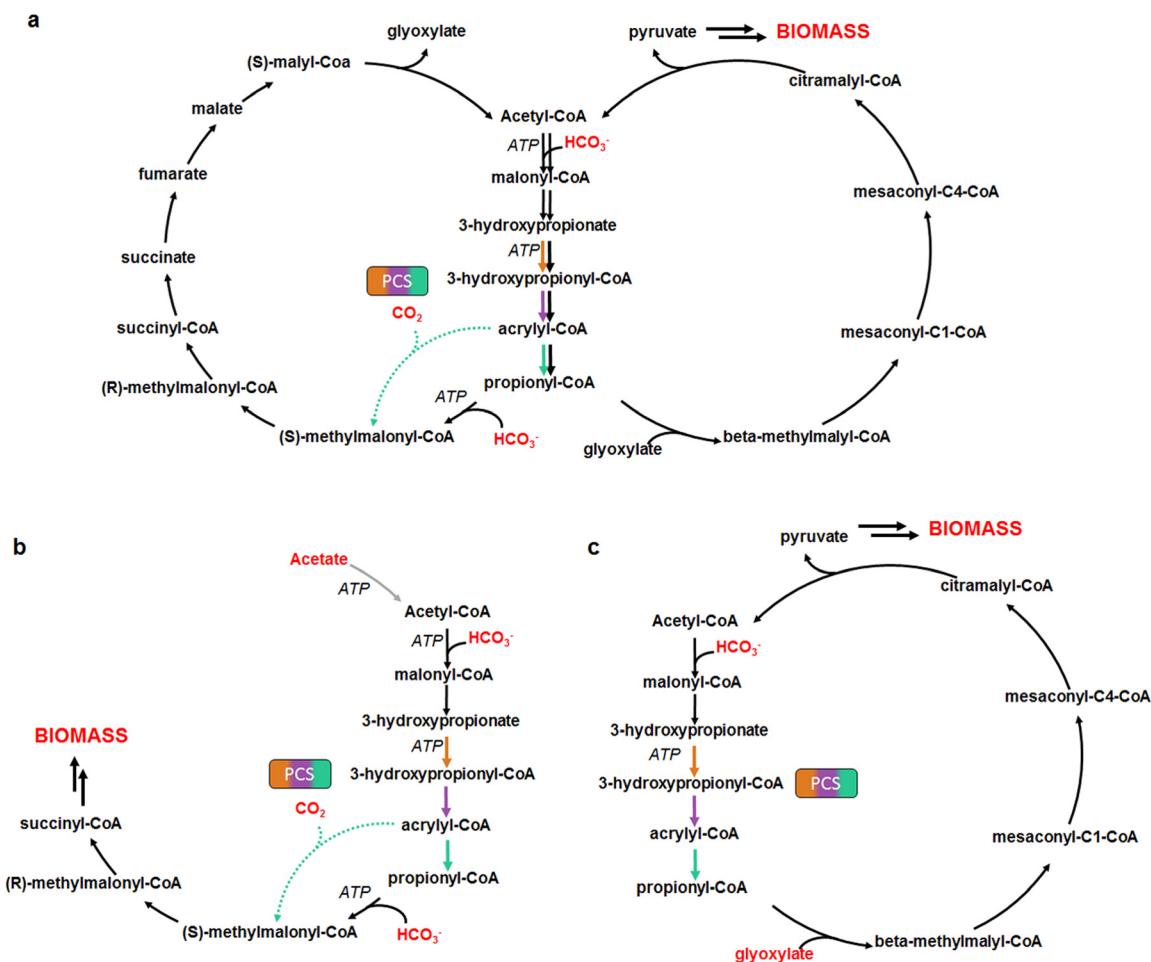


Figure 2: Functional degeneracy and multi-functionality: The different roles of the 3HP bi-cycle parts. (a) Scheme of the 3HP bi-cycle for autotrophic CO₂ fixation as described in *C. aurantiacus*. (b) Scheme of the part cycle active in acetyl-CoA assimilation, as proposed for aerobic anoxygenic phototrophs, and *C. aurantiacus* under autotrophic growth conditions in the presence of acetate. (c) Scheme of the part cycle as active in glyoxylate assimilation as proposed for *A. phosphatis* and used in the design of a synthetic photorespiration pathway. The three reactions catalyzed by the key enzyme Pcs are highlighted in orange, purple and green, respectively. The reaction of Pcs with CO₂, which was described recently to take place at low rates, is shown in dotted lines.

bacteria are necessary to maintain the redox homeostasis during phototrophy (Bill et al. 2017; Kobližek et al. 2003; Tomasch et al. 2011). A similar redox-balancing role has been ascribed to the 3HP bi-cycle before (Zarzycki and Fuchs 2011), and it might be worthwhile to speculate that the rudimentary 3HP bi-cycle fulfills this function in AAP bacteria, which account for up to 15% of the total microbial community in the upper ocean (Kobližek 2015; Kolber et al. 2001; Yutin et al. 2007).

The second cycle of the 3HP bi-cycle has not yet been assigned a function in nature (Figure 2c). However, this route has been proposed as potential propionyl-

CoA assimilatory pathway in *Candidatus Accumulibacter phosphatis* (Zarzycki and Fuchs 2011) and moreover as synthetic photorespiration bypass in cyanobacteria (Shih et al. 2014). Because this synthetic pathway would allow the additional fixation of CO₂ compared to the canonical photorespiration bypass, the 3HP bi-cycle photorespiration bypass is predicted to increase photosynthetic yield, if successfully realized *in vivo*. Altogether, these findings demonstrate how individual metabolic pathways, such as the 3HP bi-cycle, can serve multiple functions, which can be exploited by evolution and synthetic biology to increase biochemical diversity in central carbon metabolism.

Functional degeneracy in glycolysis: the Entner-Doudoroff pathway and its surprising distribution

After the glycolytic reaction sequence that later became known as the EMPP was deciphered in the first half of the 20th century, Entner and Doudoroff reported another catabolic pathway for glucose in 1952 (Entner and Doudoroff 1952). Their experiments were conducted in cell-free extracts of a *Pseudomonas* strain. A later study found that the key enzymes of this pathway, 6-phosphogluconate dehydratase (Edd) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Eda), mainly occurred in gram-negative bacteria (Kersters and De Ley 1968). When this finding was re-evaluated 24 years later, numerous gram-positive bacteria, some archaea, and a few single-celled eukaryotes could be added to the growing list of organisms utilizing the Entner-Doudoroff pathway (EDP) (Conway 1992). In the past decade, the wide distribution and metabolic versatility of the EDP has been underscored further by several findings.

One key discovery was the relevance of the EDP in photosynthetic organisms. After it was found that the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* encode the EDP, it was shown that transcript abundance of the key enzyme Eda increased strongly in *P. tricornutum* upon incubation in the dark (Fabris et al. 2012). Another condition that induces flux through the EDP in *P. tricornutum* was mixotrophic growth with glucose in the light (Zheng et al. 2013). Similarly, the Antarctic sea-ice diatom *Fragilaropsis cylindrus* showed increased expression of EDP genes in prolonged phases of darkness (Kennedy et al. 2019). Both Cyanobacteria and plants were also shown to utilize the EDP, especially under mixotrophic conditions and in a light-dark-regime (Chen et al. 2016). This pathway also enabled rapid resuscitation of the cyanobacterium *Synechocystis* sp. PCC6803 from dormancy induced by nitrogen limitation; to resume growth, both the EDP and the oxidative pentose phosphate cycle operate to catabolize cellular glycogen reserves (Doello et al. 2018). The use of the EDP, which is energetically less efficient than the EMPP (Figure 3), but at the same time also requires fewer resources for the synthesis of pathway-related proteins (Flamholz et al. 2013), might be advantageous in situations when ATP or reducing equivalents need to be generated fast (Kramer and Evans 2011; Molenaar et al. 2009), i.e., when photosynthesis is not possible anymore.

In addition to the discovery that the EDP plays a relevant role in many photosynthetic organisms, it was also

shown that the EDP is the main glycolytic strategy in marine bacteria that use glucose (Klingner et al. 2015). Out of 25 marine bacteria that were tested, 90% relied on the EDP for glycolysis. In contrast, the EMPP was used by the majority of terrestrial isolates that were tested. Use of the EDP for glycolysis is linked to higher oxidative stress tolerance, as less NADPH is consumed by the EDP (Klingner et al. 2015). Therefore, organisms relying on the EDP have an excess of the reducing metabolite NADPH at their disposal, which can then be re-allocated to mitigate oxidative stress effectively (Storz and Imlay 1999). For marine microorganisms, carbon sources such as glucose are of limited and punctual availability only. In line with the idea that the EDP requires fewer resources for the synthesis of pathway-related proteins (see above), it is tempting to speculate that the EDP might be of advantage for marine bacteria. One notable exception to this trend in marine bacteria are members of the *Vibrionaceae* (Klingner et al. 2015; Long et al. 2017). Several *Vibrio* strains are known to be important in the degradation of chitin (a derivative of glucose) and can gain an advantage over competitors by attaching to this compound via filamentation (Wucher et al. 2019). This might explain why *Vibrio* bacteria can afford to utilize the resource-intensive, but energy-efficient EMPP for glucose catabolism.

Taken together, the studies summarized here indicate a much higher ecological relevance of the EDP than previously assumed, considering that it is used both by abundant phototrophs under mixotrophic and diurnal conditions, and by about 90% of marine bacteria.

Functional degeneracy in acetyl-CoA assimilation in bacteria and archaea

Many organic compounds, including fatty acids, alcohols, and waxes, are initially metabolized to acetyl-CoA before entering central carbon metabolism. Because acetyl-CoA is completely oxidized in the TCA cycle, its assimilation into biomass requires a specialized, anaplerotic reaction sequence. For almost 50 years, the glyoxylate cycle with its two key enzymes isocitrate lyase (Icl) and malate synthase (Ms) has been the only acetyl-CoA assimilation pathway known (Kornberg and Krebs 1957). However, many bacteria had been described that do not encode Icl and thus must rely on an alternative anaplerotic pathway. The ethylmalonyl-CoA pathway (EMCP) was discovered in the Alphaproteobacterium *Rhodobacter sphaeroides* (Erb et al. 2007), and shortly afterward was confirmed to also be

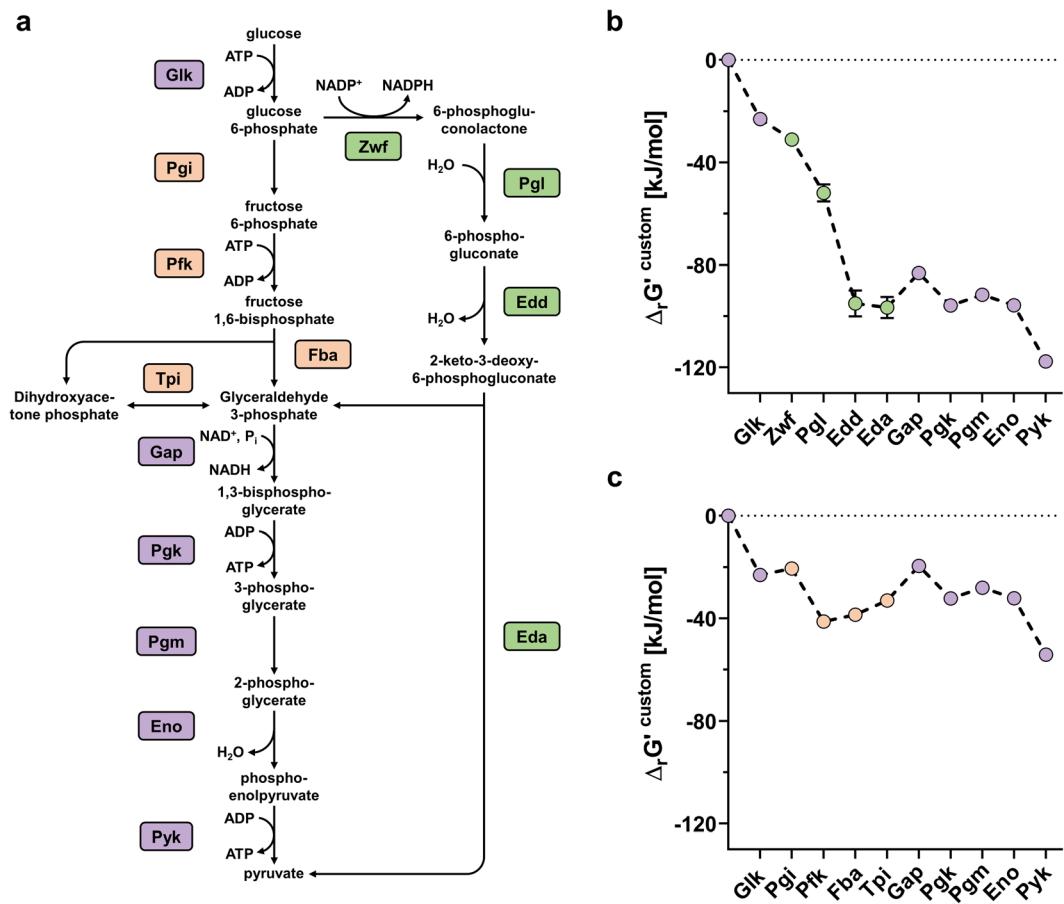


Figure 3: Comparison of the EDP and the EMPP for glucose catabolism. (a) The topologies of these glycolytic pathways. (b, c) The Gibbs free energy profiles of these pathways (b: EDP, c: EMPP). The Gibbs free energy profiles are based on calculations using the eQuilibrator software (Flamholz et al. (2012); <http://equilibrator.weizmann.ac.il>). Shown are the summarized Gibbs free energies along the individual reactions for $\Delta_f G'$ at pH 7.0, ionic strength $I = 0.1$, and assuming the following metabolite concentrations: substrates and products = 1 mM; ATP = 5 mM; ADP = 0.5 mM; NADH/NADPH = 0.1 mM; NAD⁺/NAD⁺ = 1 mM; P_i = 10 mM; H_2O = 55 M. Enzymes are abbreviated as follows: Glk – glucokinase; Pgi – glucose-6-phosphate isomerase; Pfk – 6-phosphofructokinase; Fba – fructose-bisphosphate aldolase; Tpi – triosephosphate isomerase; Zwf – glucose-6-phosphate 1-dehydrogenase; Pgl – 6-phosphogluconolactonase; Edd – 6-phosphogluconate dehydratase; Eda – 2-keto-3-deoxy-6-phosphogluconate aldolase; Gap – glyceraldehyde-3-phosphate dehydrogenase; Pgk – phosphoglycerate kinase; Pgm – phosphoglycerate mutase; Eno – enolase; Pyk – pyruvate kinase.

responsible for anaplerosis of the serine cycle in the methylotrophic *Methylobacterium extorquens* (Peyraud et al. 2011; Schneider et al. 2012; Smejkalova et al. 2010).

Surprisingly, the two functionally degenerate pathways, the glyoxylate cycle and the EMCP, are both present in the Alphaproteobacterium *Paracoccus denitrificans*. A recent study demonstrated that indeed both pathways play an active part in acetate assimilation (Kremer et al. 2019). While the EMCP seems to be constitutively expressed during growth on many different carbon sources, expression of the glyoxylate cycle is specifically induced by acetate. Neither a *Δacl* deletion strain, nor a functional knockout of the EMCP (*Δccr*) alone are lethal on acetate. However, the individual knockouts suggest that each pathway confers distinct advantages. The EMCP alone

(*Δacl*) apparently increases the growth yield, while the glyoxylate cycle (*Δccr*) allows faster growth on acetate. Genomic analyses suggest that the EMCP is the default acetate assimilation pathway in the genus *Paracoccus*, and that the glyoxylate cycle might have been acquired by HGT (Kremer et al. 2019).

When comparing the two pathways, several differences become obvious (Table 1): the glyoxylate cycle generates reducing equivalents, while the EMCP consumes them and also requires additional ATP. However, the EMCP co-assimilates two molecules of CO_2 , thus increasing biomass yield through incorporation of inorganic carbon. Moreover, the glyoxylate cycle relies on only two enzymes, while the EMCP employs a total of 13 enzymes and features several CoA-bound intermediates. The EMCP also allows

Table 1: Comparison of acetyl-CoA assimilation pathways.

Acetyl-CoA assimilation pathway	Number of enzymes	Reducing equivalents	Energy carriers	CO ₂ equivalents
Glyoxylate cycle	2 ^a	+1 NADH +1/2 FADH ₂	0 ATP (+1 CoA)	0 CO ₂
EMCP	13	-2/3 NADPH +1/3 QH ₂	-1/3 ATP (+2/3 CoA)	-1/3 CO ₂ -1/3 HCO ₃ ⁻
Methylaspartate cycle	10 ^a	-1/2 NADPH +1 NADH +1/2 FADH ₂	0 ATP (+1 CoA)	0 CO ₂

A positive sign denotes the generation (or release) of the given compound, while a negative sign denotes its consumption. The values given in the table are calculated per molecule of assimilated acetyl-CoA. Specifically, the pathways have been considered from acetyl-CoA to malate for the glyoxylate and methylaspartate cycle (including the regeneration of oxaloacetate) and to malate and succinyl-CoA for the EMCP.

^aIn addition to 5 or 8 TCA cycle enzymes for the glyoxylate or the methylaspartate cycle, respectively.

the direct assimilation of propionate and several dicarboxylic acids and might therefore serve as multi-purpose pathway to its host during various environmental conditions, while the glyoxylate cycle is specifically switched on when acetate is available (Kremer et al. 2019). Hence, the glyoxylate cycle might represent a specific adaption to acetate that allows for fast growth rates, while the versatility of the EMCP might justify the investment of cellular resources, adding a new facet to the aforementioned trade-off between protein synthesis costs, energetic efficiency, and multi-purpose use of a pathway.

Some halophilic archaea possess yet another route for acetate assimilation. *Halococcus marismortui* is able to grow on acetate as sole carbon source, despite the absence of the genes required for a functional glyoxylate cycle or EMCP. It was shown that this organism uses the so-called methylaspartate cycle (Khomyakova et al. 2011). This route is of particular interest from an evolutionary perspective, since many of its enzymes are known to participate in other metabolic pathways and seem to have been acquired by several events of HGT to tinker this pathway together. It was proposed that the methylaspartate cycle mainly serves in the utilization of polyhydroxyalkanoate-derived carbon, whereas the glyoxylate cycle (that is used by other halophilic archaea) might be responsible for growth on carbon sources metabolized via acetyl-CoA (Borjian et al. 2016). In terms of energy demand and cofactor requirements, the two pathways are comparable (Table 1). In summary, above examples demonstrate a surprising and long-overlooked functional degeneracy in acetyl-CoA

assimilation and propose distinct advantages for one or the other pathway in the context of its respective host cell.

Rediscovering functional degeneracy in glyoxylate assimilation: the β -hydroxyaspartate cycle and its distribution

Compared to acetate, the C2 molecules glyoxylate and glycolate are less prominent carbon sources for bacterial growth. However, these molecules are quite abundant by themselves, due to the fact that glycolate is released on a gigatonne scale by aquatic phototrophs (Wright and Shah 1977). Furthermore, glyoxylate is also formed as breakdown product of allantoin and purine bases (Vogels and Vanderdrift 1976), as well as nitrilotriacetate (NTA) and ethylenediaminetetraacetate (EDTA) (Bohuslavek et al. 2001; Liu et al. 2001).

While the dicarboxylic acid cycle can serve to oxidize glyoxylate to carbon dioxide (Kornberg and Sadler 1960), two different pathways for the net assimilation of glyoxylate into biomass are known (Figure 4a, b). Both the glycerate pathway (Hansen and Hayashi 1962; Krakow and Barkulis 1956) and the β -hydroxyaspartate cycle (BHAC) (Kornberg and Morris 1963) were first investigated in the 1950s/60s. However, while glyoxylate carboligase and tartronate semialdehyde reductase, the key enzymes of the glycerate pathway, were identified quickly, probably because they are encoded by the model organism *E. coli* (Goto and Kornberg 1961; Gupta and Vennesland 1964), the BHAC and its key enzyme iminosuccinate reductase were fully described only in 2019 (Schada von Borzyskowski et al. 2019). Both of these pathways funnel two molecules of glyoxylate into central metabolism. Yet, there are distinct differences between the two functionally degenerate pathways: the BHAC is not only energetically more efficient than the glycerate pathway, it is also carbon-neutral: while the glycerate pathway releases one molecule of carbon dioxide in the assimilation process, the BHAC does not. Refixation of CO₂ by PEP carboxylase is generally possible, but linked to additional enzyme synthesis costs. However, the thermodynamic profile for the glycerate pathway is more favorable under standard conditions than for the BHAC, driving the pathway efficiently towards assimilation (Figure 4c). While marker genes of the BHAC are almost 20-fold more abundant in marine metagenomes than marker genes of the glycerate pathway (Schada von Borzyskowski et al. 2019), the latter metabolic

route seems to be more prevalent in bacterial isolates in general (Figure 4d).

Glyoxylate also needs to be assimilated during photorespiration to avoid toxic effects of this reactive aldehyde on the cell. For this purpose, cyanobacteria, algae, and plants use either the glycerate pathway or the glycine cleavage

complex (Eisenhut et al. 2008; Hagemann et al. 2016). Alternatively, glyoxylate can be co-assimilated with acetyl-CoA by malate synthase or completely oxidized to CO_2 (Eisenhut et al. 2008; South et al. 2019). Interestingly, the BHAC is present in the genomes of some Proteobacteria that also encode the CBB cycle (Schada von Borzyskowski

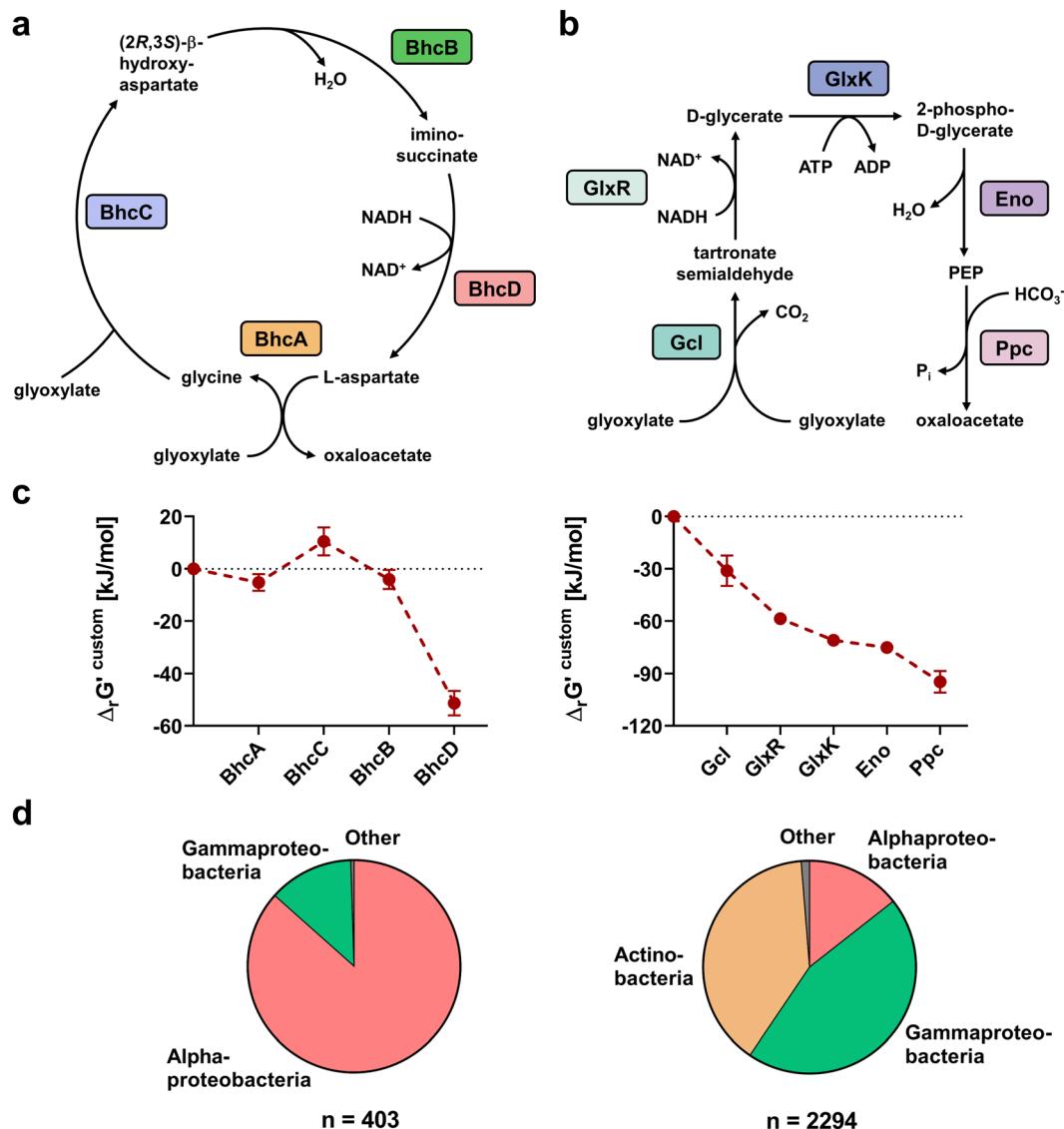


Figure 4: Comparison of net glyoxylate assimilation pathways. The topologies of the BHAC (a) and the glycerate pathway (b) are shown. (c) The Gibbs free energy profiles of these pathways (left: BHAC, right: glycerate pathway). The Gibbs free energy profiles are based on calculations using the eQuilibrator software (Flamholz et al. (2012); <http://equilibrator.weizmann.ac.il>). Shown are the summarized Gibbs free energies along the individual reactions for $\Delta_f G^\circ$ at pH 7.0, ionic strength $I = 0.1$, and assuming the following metabolite concentrations: substrates and products = 1 mM; iminosuccinate = 0.01 mM; ATP = 5 mM; ADP = 0.5 mM; NADH = 0.1 mM; NAD⁺ = 1 mM; CO_2 = 0.01 mM; P_i = 10 mM; H_2O = 55 M. (d) Phylogenetic assignment of BhcC (left) and Gcl (right) in sequenced bacterial genomes. KEGG orthology numbers K18425 and K01608 were searched using the software AnnoTree (Mendler et al. (2019); <http://annotree.uwaterloo.ca>) with the following parameters: 60% identity, E value 1e-20, 70% subject and query alignment. Out of 403 bacterial strains that encode a homolog of BhcC in their genome, 304 (=75.4%) were sampled from marine environments. Enzymes are abbreviated as follows: BhcA – aspartate-glyoxylate aminotransferase; BhcB – β -hydroxyaspartate dehydratase; BhcC – β -hydroxyaspartate aldolase; BhcD – iminosuccinate reductase; Gcl – glyoxylate carboxylase; GlxR – tartronate semialdehyde reductase; GlxK – glycerate kinase; Eno – enolase; Ppc – phosphoenolpyruvate carboxylase.

et al. 2019). However, its direct involvement in photorespiration in these microorganisms still awaits experimental validation. The apparent lack of photosynthetic organisms that employ the BHAC in photorespiration is surprising. The observation that the most efficient natural glyoxylate assimilation pathway is apparently not used during photorespiration is reminiscent of the fact that enoyl-CoA carboxylases/reductases (ECRs), the most efficient CO₂-fixing enzymes (Erb 2011), are not used in natural carbon fixation pathways, but only in synthetic CO₂ fixation pathways so far (Schwander et al. 2016).

Genetic redundancy and functional degeneracy of methylamine utilization modules: distinct roles in carbon and nitrogen metabolism

The assimilation of C1 compounds requires specialized pathways to utilize these molecules as building blocks for central metabolism. In the case of methylamine, a simple organic amine that can serve as both a carbon and a nitrogen source, an interesting dichotomy of metabolic routes was described in methylotrophic bacteria. Methylamine can be deaminated by the periplasmic enzyme methylamine dehydrogenase (MaDH), with the resulting formaldehyde being further utilized by C1 metabolic pathways. This enzyme (Eady and Large 1968) and its genes (Chistoserdov et al. 1991) were discovered in the model methylotroph *M. extorquens* AM1. An alternative route is the N-methylglutamate (NMG) pathway, in which methylamine is first linked to glutamate. This pathway also produces formaldehyde, but requires three cytoplasmic enzymes, and generates one reducing equivalent while consuming one ATP (Latypova et al. 2010; Nayak et al. 2016). Curiously, many methylotrophs encode the enzymes for both of these pathways (Nayak and Marx 2015), but the NMG pathway is not able to rescue growth on methylamine of a MaDH deletion strain of *M. extorquens* AM1 (Chistoserdov et al. 1994). Using deletion mutants and experimental evolution, two distinct roles could be ascribed to the two methylamine oxidation modules: while MaDH facilitates rapid growth on methylamine as carbon source, the NMG pathway enables nitrogen assimilation from methylamine, but only allows slow growth on this substrate (Nayak et al. 2016). This result is further supported by heterologous expression of MaDH in *M. extorquens* PA1, which only encodes the enzymes for the NMG pathway.

Transplantation of MaDH, which also seems to occur frequently via HGT in nature, lead to a fivefold increase in growth rate (Nayak and Marx 2015).

Interestingly, a very similar paradigm was found for methylamine utilization by the archeal methanogen *Methanosarcina acetivorans*. While one methyltransferase paralog is necessary for methanogenesis from methylamine, a second paralog enables the use of this compound as nitrogen source (Nayak and Metcalf 2019).

Conclusions

For many years, microbiological research has focused on selected model organisms. While we have gained a deep understanding of their metabolism, this exclusive focus has prevented us to appreciate the full diversity of microbial metabolism. However, the transition into a (meta-)genomics era has opened our eyes and revealed a much more complex picture of microbial metabolism and its intricacies. This has not only lead to the discovery of new pathways for existing metabolic traits—including the EMCP and the methylaspartate cycle for acetyl-CoA assimilation, or the BHAC for glyoxylate assimilation—but also drew our attention to (parts of) well-known pathways that are used for other, unexpected metabolic traits, such as the partial 3HP bi-cycle in heterotrophic AAP bacteria.

Furthermore, the ever-growing number of sequenced (meta-)genomes allows us to draw conclusions about the ecological distribution and evolutionary origin of metabolic pathways. This is of particular interest in the case of functional degeneracy in an organism, e.g., the co-occurrence of the CBB and rTCA cycles for autotrophic CO₂ fixation, or the glyoxylate cycle and EMCP for acetyl-CoA assimilation. In above examples, genetic redundancy and functional degeneracy might have seemed unnecessary and a curiosity of evolution. However, recent studies revealed that they indeed exist for a reason. It will therefore be indispensable to continue using biochemistry, enzymology, and growth assays to complement and interpret the overwhelming (meta-)genomics data. In some cases, such as the rTCA cycle, it will actually be impossible to predict the function of a given pathway simply by bioinformatics approaches. It can therefore be expected that more surprises will be revealed when the vast amount of gene sequences will be translated into biochemical and functional knowledge by scientists in the coming years. While anything found to be true of *E. coli* probably still is true of elephants, it might well be different in another microorganism.

Note: This study follows GTDB taxonomy (Parks et al. 2018).

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Conflict of interest statement: The Max-Planck-Gesellschaft zur Förderung der Wissenschaften is the patent applicant for the following three patents. All patent applications are pending. L.S.v.B. and T.J.E. have filed European patent no. EP 19190404.4 for the production of plants with altered photorespiration due to implementation of the BHAC. L.S.v.B. and T.J.E. have filed European patent no. EP 18167406.0 for the production of photoautotrophic organisms with altered photorespiration due to implementation of the BHAC. L.S.v.B. and T.J.E. have filed European patent no. 18211454.6 for the enantioselective preparation of primary amine compounds using the enzyme BhCD or its homologues.

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