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Research review paper

Optimizing microbial networks through metabolic bypasses

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ABSTRACT

Metabolism has long been considered as a relatively stiff set of biochemical reactions. This somewhat outdated and dogmatic view has been challenged over the last years, as multiple studies exposed unprecedented plasticity of metabolism by exploring rational and evolutionary modifications within the metabolic network of cell factories. Of particular importance is the emergence of metabolic bypasses, which consist of enzymatic reaction(s) that support unnatural connections between metabolic nodes. Such novel topologies can be generated through the introduction of heterologous enzymes or by upregulating native enzymes (sometimes relying on promiscuous activities thereof). Altogether, the adoption of bypasses resulted in an expansion in the capacity of the host's metabolic network, which can be harnessed for bioproduction. In this review, we discuss modifications to the canonical architecture of central carbon metabolism derived from such bypasses towards six optimization purposes: stoichiometric gain, overcoming kinetic limitations, solving thermodynamic barriers, circumventing toxic intermediates, uncoupling product synthesis from biomass formation, and altering redox cofactor specificity. The metabolic costs associated with bypass-implementation are likewise discussed, including tailoring their design towards improving bioproduction.

1. Introduction

Cell factories are key drivers of industrial biotechnology by converting renewable substrates into bio-based chemicals. Optimizing their metabolic networks enables consolidation of existing bioprocesses (*e.g.* by increasing product formation), as well as to explore new-to-industry ones. Many intracellular conversions involved in substrate-to-product transformation form part of the central metabolism of microorganisms. Naturally, the central metabolism is geared to provide the essential energy and building blocks for biomass growth, in an architecture that is often described as a 'bow-tie' structure (Nielsen and Keasling, 2016) (Fig. 1). This metaphor indicates that multiple substrates can enter the metabolic network in several entry points, potentially generating a wide array of products from a set of metabolic intermediates. The 12 universal biomass precursors, which must be generated for biomass buildup, lay at the very core of this architecture (Fig. 1) (Neidhardt et al., 1990). Hence, the set of reactions supporting the synthesis of biomass precursors is highly conserved across the tree of life. This is organized in a 'minimal walk' principle that minimizes the energy investment for protein synthesis while maximizing cellular economics (Noor et al., 2010). Most of industrially relevant products are derived from at least one of these key biomass precursors (Fig. 1).

Traditional metabolic engineering strategies for increasing product formation are mostly embedded within this minimal walk, with approaches that aim at improving the carbon flux within the boundaries of the original metabolic architecture of the host. These include, *e.g.*, improving precursors- or cofactors-supply by eliminating by-product formation, or overexpressing genes of rate-limiting enzymes (Davy et al., 2017). Nevertheless, several alternative routes to this minimal walk have either been characterized in nature or designed *de novo*. These linkages, which we refer to as metabolic 'bypasses' (Fig. 2), expand the metabolic topology of a cell factory, ultimately influencing important production parameters such as titers, rates and yields (TRY values).

This review aims at presenting the design principles of metabolic bypasses by illustrating how they have been implemented in cell factories (Fig. 2). For this purpose, we summarize the most relevant examples, with a special focus on the ones that have been established *in vivo*. Moreover, we reasoned that these novel biochemical connections optimize microbial metabolism by satisfying at least one of six general optimization goals: achieving stoichiometric gain, avoiding kinetic

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limitations and unfavorable promiscuities, improving the thermodynamic drive of one or more reactions, circumventing cytotoxic intermediates, uncoupling precursor supply routes from biomass for enhancing product synthesis, and altering the redox cofactor type (Fig. 2). Altogether, the access of bypasses to the toolbox of metabolic engineers and synthetic biologists increases the possible design-space to support metabolic optimizations for sustainable bioproduction.

2. Cell factories improvement using metabolic bypasses

Here, we present a selection of relevant examples of metabolic bypasses reported in literature and summarized in Table 1. Rather than comprehensively listing all the -already extensive- literature on this topic, our intention is to describe their diversity in applications within the scope of optimizing a microbial cell factory. Engineering of metabolic bypasses can be motivated by six different purposes, each resulting in a specific optimization goal. We therefore organized the manuscript following such a structure. As some of the demonstrated metabolic bypasses can tackle multiple obstacles at once, they will be considered in more than one category.

2.1. Achieving stoichiometric gain

This first design principle consists of bypass implementation for improving reaction stoichiometries. More precisely, this category describes rewiring efforts aimed to increase product yields by improving carbon-conservation in the metabolic conversion. This approach has been widely used in central and secondary metabolism for both generation of biomass precursors and synthesis of target products. Some of the bypasses presented in this section have also been extensively covered in two valuable reviews focusing on holistic bioengineering approaches (Aslan et al., 2017) and CO_2 recycling pathways (François et al., 2020).

2.1.1. Carbon conservation within central metabolism

Glycolysis is the most studied biochemical route (Bar-Even et al., 2012; Fothergill-Gilmore and Michels, 1993; Fuhrer et al., 2005; Grüning and Ralser, 2021; Romano and Conway, 1996), and several variants of this core catabolic pathway have been described in literature for all domains of life. Overall, glycolysis catabolizes glucose to pyruvate, while generating six of the 12 essential biomass precursors (glucose 6-phosphate, fructose 6-phosphate, glyceraldehyde 3-phosphate, glycerate 3-phosphate, phosphoenolpyruvate, pyruvate) and providing energy by regenerating ATP and redox cofactors.

A recent review discussed the different overarching approaches that have been covered for engineering glycolysis within cell factories (Kopp and Sunna, 2020). Under aerobic conditions, glycolysis is usually followed by the decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA) *via* the pyruvate dehydrogenase complex (PDH). This redox conversion harvests reducing power in the form of NADH. Anaerobically, pyruvate conversion to acetyl-CoA is catalyzed in *Escherichia coli* by the

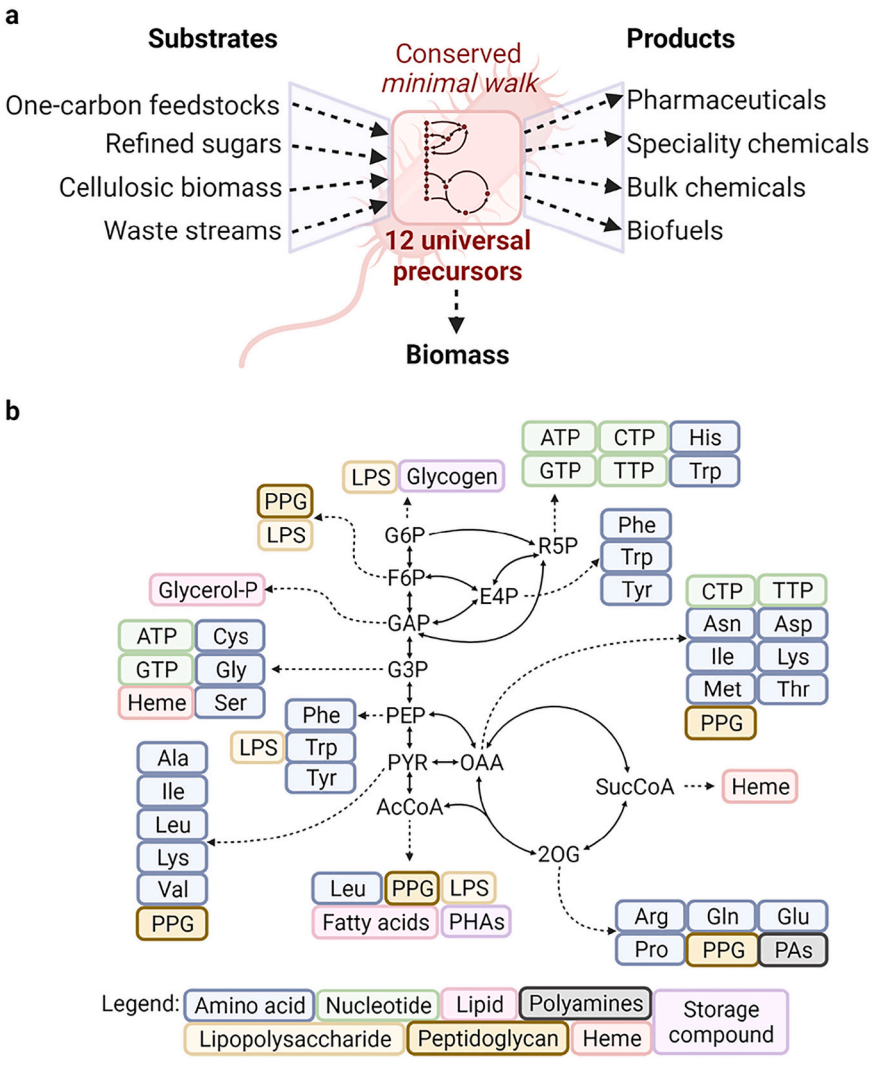

Fig. 1. Bow-tie structure of metabolism arranged in a minimal walk. a) The scheme shows the broad range of substrates that can be accepted and products that can be generated by cell factories. At the core lays the central metabolic network, which is organized in a 'minimal walk' (Noor et al., 2010) to support the biosynthesis of the 12 universal biomass precursors. Often, these are also key intermediates in the conversion of substrates into value-added products. b) Overview of the bacterial (*Escherichia coli*) biomass building blocks that stem from the 12 universal biomass precursors of central carbon metabolism. Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; R5P, ribose 5-phosphate; E4P, erythrose 4-phosphate; GAP, glyceraldehyde 3-phosphate; G3P, glycerate 3-phosphate; PEP, phosphoenol-pyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; SucCoA, succinyl-CoA; 2OG, 2-oxoglutarate. Created with BioRender.com.

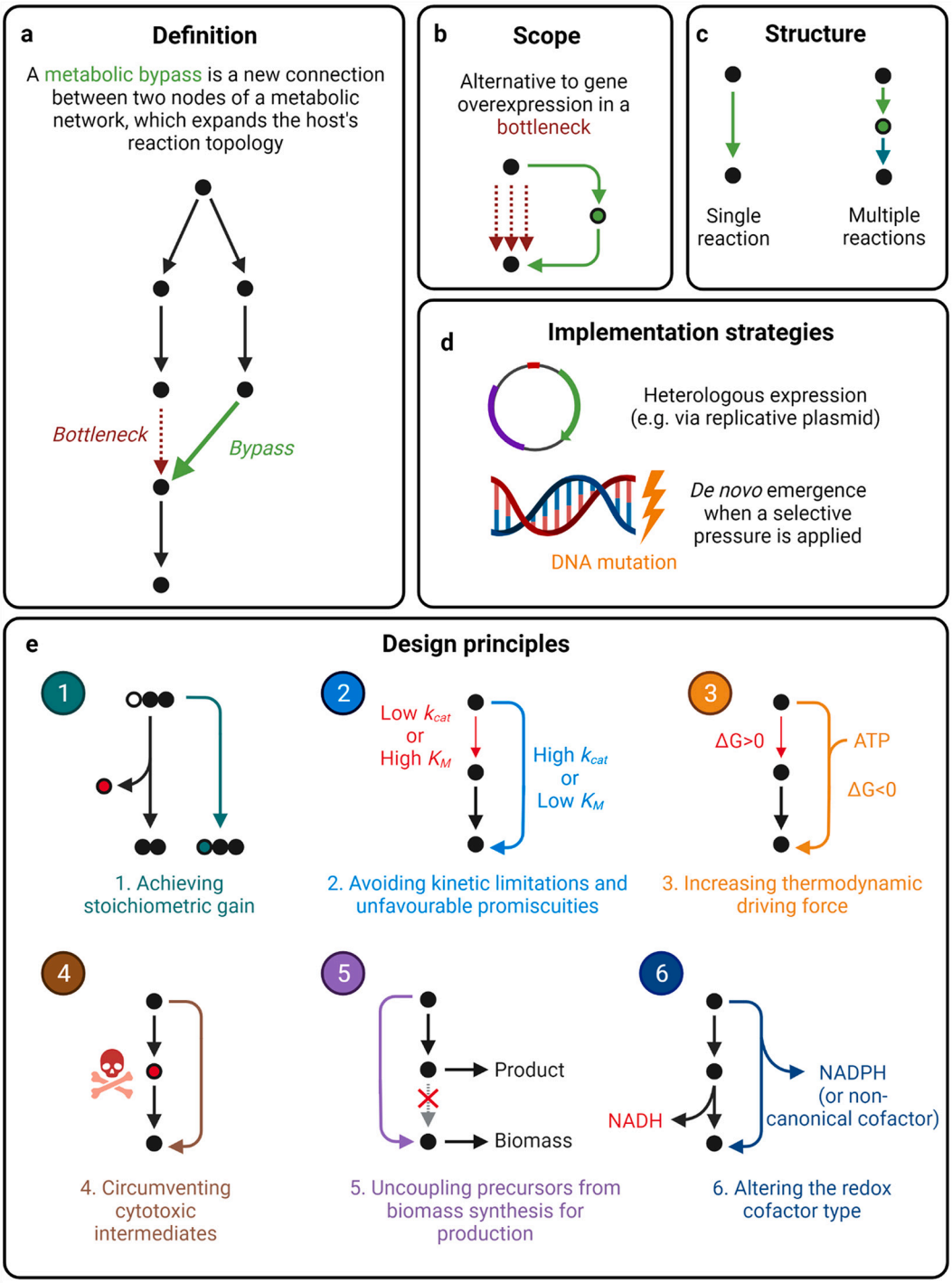

Fig. 2. The concept of metabolic bypass presented in this review. a) We define as metabolic bypass a novel connection introduced between two existing nodes of the metabolic network, which expands the metabolic topology of the host. b) A bypass represents an alternative to the classic approach of gene overexpression. In fact, carbon metabolism can present bottlenecks that might be difficult to overcome by simply increasing the number of rate-limiting enzyme(s). Under these circumstances, metabolic bypasses constitute a tool available to the designer to optimize carbon flux towards a product of interest. c) Such a novel link is the result of the activity of at least one enzyme, but can be established also *via* the combination of several enzyme activities, which could come from different organisms through a 'mix and match' approach (Erb et al., 2017). d) Bypasses can be introduced rationally *via* heterologous gene expression from non-native routes, or they can emerge *de novo*. When a selective pressure is applied (*e.g.* deletion of a key metabolic reaction), a native silent gene gets activated or a generalist enzyme can evolve to expand its substrate acceptance range, thereby replacing the missing catalytic activity. e) We pose that implementing bypasses satisfies up to six metabolic design principles: achieving stoichiometric gain; avoiding kinetic limitations and unfavorable promiscuities; increasing thermodynamic driving force; circumventing cytotoxic intermediates; uncoupling precursors from biomass synthesis for bioproduction; altering the redox cofactor type. Often, a bypass fulfils multiple optimization purposes at once. Created with BioRender.com.

Table 1
Overview of the most relevant bypasses discussed in this review.

	Design principle							
Bypass	Stoichiometry[a]	Kinetic[b]	Thermodynamic[c]	Toxic intermediates[d]	Alternative precursor supply[e]	Cofactor type[f]	Organism	Reference
Bifido shunt (Pkt)[g]	+						*C. glutamicum, E. coli, S. cerevisiae*	(Chinen et al., 2007; Krüsemann et al., 2018; Meadows et al., 2016; Papini et al., 2012)
Non-oxidative glycolysis (NOG)[g]	++						*E. coli*	(Bogorad et al., 2013)
Reverse glyoxylate shunt (rGS)[g]	++		+				*E. coli*	(Mainguet et al., 2013)
Weimberg pathway[g]	++				++		*C. glutamicum, E. coli, P. putida*	(Bator et al., 2020; Lu et al., 2021; Radek et al., 2014)
Dahms pathway[g]	++						*E. coli, P. putida*	(Bator et al., 2020; Choi et al., 2016)
Xylulose-1-phosphate (X1P)[g]	++			–			*E. coli*	(Cam et al., 2016)
Ribulose 1-phosphate (R1P)	++			–			*E. coli*	(Pereira et al., 2016)
Rubisco shunt[g]	+++						*E. coli, S. cerevisiae*	(Guadalupe-Medina et al., 2013; Li et al., 2015; Papapetridis et al., 2018; Tseng et al., 2018)
Gnd-Entner-Doudoroff (GED) shunt[g]	+++						*E. coli*	(Satanowski et al., 2020)
RuMP shunt	+++			–			*E. coli*	(Chen et al., 2018; He et al., 2018)
GapN[g]			+			+	*C. glutamicum, E. coli*	(Centeno-Leija et al., 2014; Lindner et al., 2018; Takeno et al., 2010)
Gapdh (NADH– > NADPH)			–			+	*C. glutamicum, E. coli*	(Bommareddy et al., 2014; Martínez et al., 2008)
Glycerol bypass						+	*S. cerevisiae*	(Islam et al., 2017; Klein et al., 2016)
Bypass of methylglyoxal for 1,2-PDO				+			*E. coli*	(Niu et al., 2019)
Modified serine cycle		+		+			*E. coli*	(Yu and Liao, 2018)
Phosphorylative bypass of mevalonate pathway		+		+			*E. coli*	(Kang et al., 2016)
Shikimate pathway bypass		+					*E. coli*	(Ran et al., 2004; Ran and Frost, 2007)
Bypass of Dxs for isoprenoid biosynthesis	++	+			+		*E. coli*	(King et al., 2017; Kirby et al., 2015)
Modified C4 route for 5-ALA biosynthesis		+			+		*E. coli*	(Ren et al., 2018)
Polyketoacyl-CoA thiolases bypasses	++	+	–				*E. coli*	(Tan et al., 2020)
Malonyl-CoA bypass[g]			+				*E. coli, Synechococcus elongatus, S. cerevisiae*	(Lan and Liao, 2012; Menon et al., 2015; Tippmann et al., 2017)
Homoserine cycle			+				*E. coli*	(He et al., 2020)
Isoprenoid alcohol pathway		+					*E. coli*	(Clomburg et al., 2019)
MVA – > MEP replacement[g]	+	–					*S. cerevisiae*	(Carlsen et al., 2013; Kirby et al., 2016; Partow et al., 2012)
MEP – > MVA replacement	–	+					*E. coli, R. sphaeroides*	(Martin et al., 2003; Orsi et al., 2020b, 2020c)
Glyoxylate shunt for Ethylmalonyl-CoA pathway					++		*M. extroquens*	(Schada Von Borzyskowski et al., 2018)

[a] +++: carbon positive; ++: no carbon loss; +: reduced carbon loss; -: carbon negative.
[b] +: avoids one or more kinetically challenging reactions; -: decrease in kinetic performances;
[c] +: + increased thermodynamic drive; -: reduced thermodynamic drive;
[d] +: avoids a toxic intermediate; -: requires a toxic intermediate;

[e] ++: demonstrated to increase product formation by functioning as alternative route for biomass precursor supply. +: can in principle serve as bypass for anabolic precursor, but its implementation for this purpose has not been proven yet.
[f] +: generate cofactor with more negative reduction potential.
[g] Metabolic cost: the use of this bypass results in a net decrease in ATP or redox cofactor available for the cell.

non-reducing enzyme pyruvate formate lyase (PFL), which produces formate as byproduct. Alternatively, *e.g.* in yeast, acetyl-CoA formation can also happen through a three-step reaction, first the decarboxylation of pyruvate into acetaldehyde *via* pyruvate decarboxylase (van Rossum et al., 2016). Then, the enzyme acetaldehyde dehydrogenase converts acetaldehyde into acetate, while generating NAD(*P*)H. The last reaction converts acetate into acetyl-CoA *via* acetyl-CoA synthetase and requires an investment of two ATP equivalents. Acetyl-CoA is another essential biomass precursor, as well as the fuel of the tricarboxylic acid (TCA) cycle. Moreover, it is an important precursor for a plethora of interesting biotechnological products (Lee et al., 2019). Since transition from glycolysis to acetyl-CoA results in the loss of one carbon molecule per acetyl-CoA formed, the generation of acetyl-CoA from pyruvate decarboxylation represents a stoichiometric limitation for high-yield synthesis of this compound and derivatives.

In nature, alternative glycolytic routes exist that could offer a solution to this problem. Within this group, the 'bifido shunt', firstly discovered in *Bifidobacterium bifidum* (De Vries et al., 1967), is used by *Bifidobacteria spp.* to convert hexoses into lactate and acetate at high yields (Fushinobu, 2010). The key enzyme of this shunt is phosphoketolase (PKT), which irreversibly cleaves fructose 6-phosphate (FPK activity) or xylulose 5-phosphate (XPK activity), using an additional inorganic phosphate as attacking group. In the first case, acetyl-phosphate and erythrose 4-phosphate (E4P) are generated, and in the second glyceraldehyde 3-phosphate (GAP) and acetyl-P (Fig. 3a). In *Bifidobacterium spp.*, a PKT variant is capable of accepting both xylulose 5-phosphate (XPK activity) and fructose 6-phosphate (FPK activity) (Fushinobu, 2010). Furthermore, this PKT enzyme was proven to also accept sedoheptulose 7-phosphate as substrate *in vivo* (SPK activity), generating ribose-5-phosphate (R5P) and acetyl-P as products (Krüsemann et al., 2018).

One of the first applications of PKT for metabolic engineering was the heterologous expression of the *xfp* gene from *B. animalis* encoding an enzyme with XPK activity. This was tested in *Corynebacterium glutamicum*, and bypassed the stoichiometric limitation imposed by the native Embden-Meyerhof-Parnas (EMP) pathway and PDH, increasing the yield of the TCA cycle-derived product L-glutamate (Chinen et al., 2007) (Fig. 3a). In this way, authors could generate acetyl-CoA through acetyl-P with reduced carbon loss. Thereby, the XPK-derived acetyl-CoA could replenish the TCA cycle, eventually increasing L-glutamate yields (Chinen et al., 2007). A similar rationale was followed in an isoprenoid overproducing strain of *Saccharomyces cerevisiae* (Meadows et al., 2016). Here, conversion of glucose into the isoprenoid precursor acetyl-CoA was enhanced by introducing XPK as shunt for the generation of acetyl-CoA *via* acetyl-P (Fig. 3a). Both examples share the use of XPKs as bypasses within glucose catabolism for avoiding the stoichiometric limitation of pyruvate decarboxylation to acetyl-CoA. In both cases reported, acetyl-CoA yield increased, supporting higher yields of acetyl-CoA derived compounds. Use of PKTs for metabolic engineering is not limited to these two examples, and this has been reviewed elsewhere (Henard et al., 2015).

The logic of carbon conservation through the use of PKT also formed the bases for the design of the non-oxidative glycolysis (NOG) pathway (Bogorad et al., 2013). This synthetic pathway perfectly matches the optimization principle for stoichiometric gain. NOG's architecture is based on the 'bifido shunt' and therefore relies on PKT. Additionally, it includes the non-oxidative carbon re-arrangement branch of the pentose phosphate pathway (PPP). The pathway is designed to reach the synthesis of 3 acetyl-CoA equivalents from 1 glucose consumed, instead of the 2 acetyl-CoA generated *via* the canonical EMP (Fig. 3b). This increase in carbon yield comes at the costs of zero gain in ATP and redox cofactors (compared to 2 ATP and 4 NADH generated *via* EMP). Therefore, it is an ideal pathway to exploit for the synthesis of acetyl-CoA derived compounds that do not require any further energy or redox input (*i.e.* acetate or acetone). Due to the lack of ATP and redox equivalents generated, the operation of this pathway as central glycolytic pathway may impair growth. In fact, for implementing a strain exclusively relying on the NOG, the whole central metabolic network of *Escherichia coli* was rewired by a combination of rational engineering and adaptive laboratory evolution (ALE) (Lin et al., 2018). As described by the authors of this work, the optimal setup for using such a strain is a two-phase cultivation. During the initial aerobic growth phase, biomass formation is supported by the NOG in combination to the TCA cycle, glyoxylate shunt and gluconeogenesis for the conversion of acetyl-CoA in all the 12 biomass precursors and energy required for growth. Then, a fermentation phase follows, with glucose metabolized to acetate *via* the NOG. The EMP pathway allows a maximum-theoretical yield of 2 mol of acetate per glucose (4 out of 6 carbons conserved, 67%), whereas through NOG it is possible to produce up to 3 acetate moles (6 out of 6 carbons conserved, 100%). Indeed, the engineered strain resulted in acetate yields up to 80% of the theoretical carbon-yield, which corresponds to about 4.8 out of 6 carbons conserved. Altogether, this improved carbon yield of the NOG over the EMP can be obtained exclusively during non-growing conditions, for production pathways that do not require the investment of ATP or reducing power.

Carbon conservation for avoiding the loss of CO_2 while generating acetyl-CoA was also attempted in the metabolic network downstream the pyruvate node. A reverse glyoxylate shunt was designed and implemented in *E. coli* to allow for the production of two acetyl-CoA molecules from succinate (Fig. 3c) (Mainguet et al., 2013). Naturally, the bioconversion of succinate to acetyl-CoA in *E. coli* will proceed *via* oxaloacetate (OAA), PEP and pyruvate, resulting in two decarboxylation events, yielding only one acetyl-CoA from succinate (Fig. 3c). Moreover, as we will see in the section related to thermodynamic constraints, the implementation of the reverse glyoxylate shunt required the use of ATP to circumvent irreversible reactions, which is another design principle discussed in this review. Despite being an elegant exercise of metabolic rewiring, this bypass seems to represent mainly an academic endeavor still far away from application in large-scale industrial biotechnology, since full growth required supplementing the cultivation medium with glucose, malate, and succinate. Nevertheless, if fully implemented, this pathway holds potential for, *e.g.*, synthesis at high stoichiometric yields of citrate and amino acids derived from 2-oxoglutarate (2OG) (Vuoristo et al., 2015).

Pentoses are attractive feedstocks for the bio-based economy, representing a substantial fraction of hemicellulose, a major component (15-35% *w*/w) of lignocellulosic hydrolysates (Gírio et al., 2010). Industrial workhorses like *E. coli* and *S. cerevisiae* have long been engineered and evolved for efficiently assimilating pentoses (Li et al., 2019). Pentoses are generally catabolized *via* the isomerase pathway in the PPP (Fig. 3d), and then funneled into lower glycolysis. Since pentoses contain one C-atom less than hexoses, it is desirable to avoid carbon loss while funneling the carbon flux towards biomass and product precursors. In other words, carbon-efficient catabolism of pentoses requires bypasses that permit a stoichiometric gain over the canonical metabolic network. As we will see below, there is a high degree of freedom in the applicability of different pentose degradation pathways for metabolic engineering purposes.

A stoichiometric efficient way (without decarboxylation) of converting xylose into the C5 biomass precursor 2OG is *via* the Weimberg pathway (Fig. 3d). This five-step anaplerotic pathway results in a theoretical carbon yield of 100% (5 out of 5 carbons conserved), while

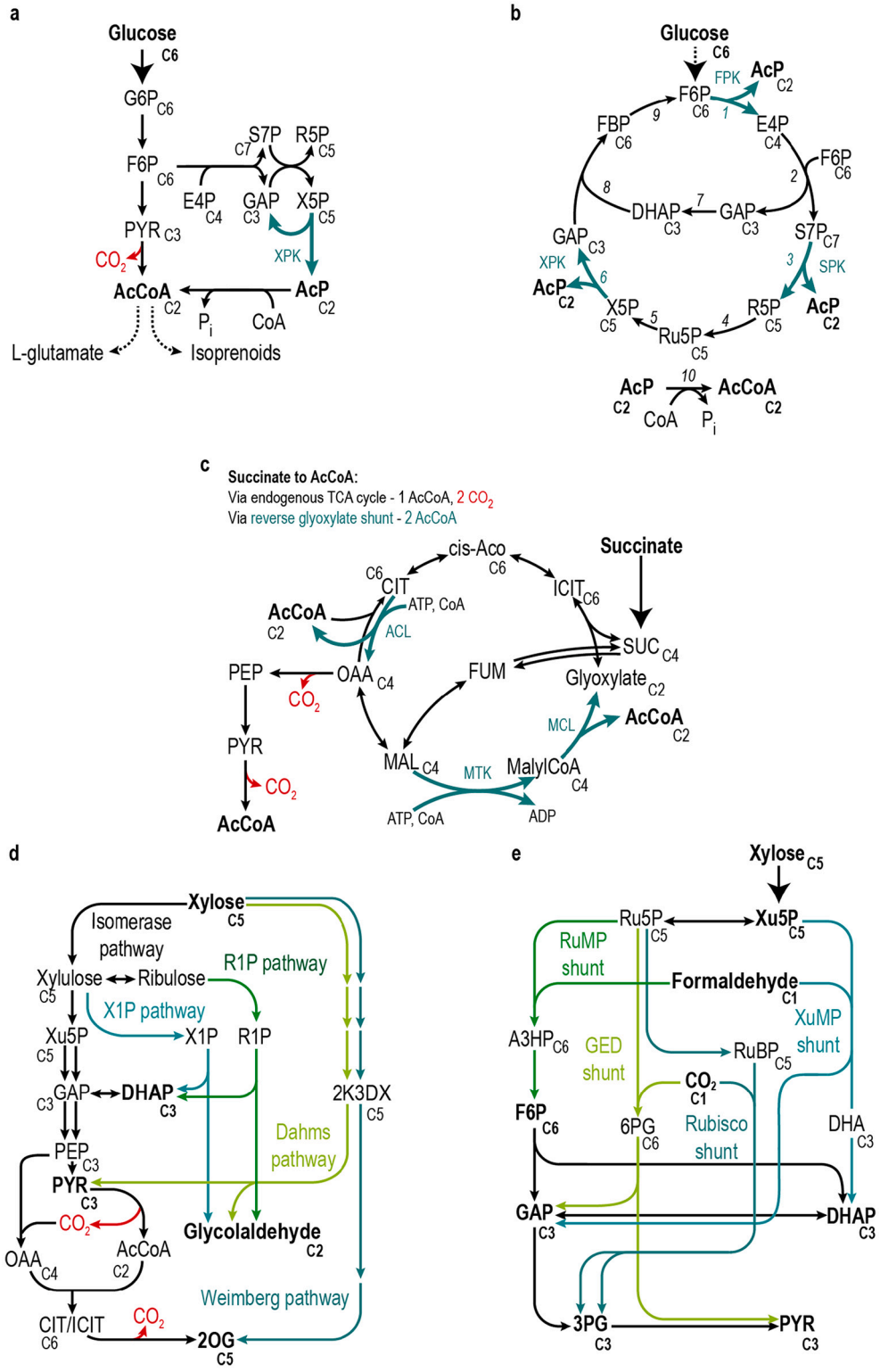

Fig. 3. Bypasses for stoichiometric gain in central carbon metabolism. a) Use of xylulose 5-phosphate phosphoketolase (XPK) circumvents carbon loss when generating acetyl-CoA (AcCoA) from pyruvate. XPK cleaves xylulose 5-phosphate (X5P) into glyceraldehyde 3-phosphate (GAP) and acetyl-phosphate (AcP). The latter can be converted *via* a phosphate acetyltransferase into AcCoA. This bypass has been reported for the production of several added-value molecules, *e.g.* L-glutamate and isoprenoids. b) The use of different phosphoketolase variants can realize glucose catabolism *via* a non-oxidative glycolysis pathway (NOG), which results in the generation of three acetyl-CoA equivalents from a single glucose molecule. This is stoichiometrically superior to most glycolytic pathways, which generate only two acetyl-CoA molecules per glucose catabolized. An example of a NOG-pathway is shown in this panel, involving three different phosphoketolase activities: fructose 6-phosphate phosphoketolase (FPK), xylulose 5-phosphate phosphoketolase (XPK) and sedoheptulose 7-phosphate (SPK). The steps of this synthetic cycle are highlighted by numbers. 1: PKT (FPK activity); 2: transaldolase; 3: PKT (SPK activity); 4: ribulose 5-phosphate epimerase; 5: ribose 5-phosphate isomerase; 6: PKT (XPK activity); 7: triosephosphate isomerase; 8: fructose-bisphosphate aldolase; 9: fructose-1,6-bisphosphatase; 10: phosphate acetyltransferase. Other abbreviations: G6P: glucose 6-phosphate; FBP: fructose 1,6-bisphosphate; GAP: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; 3PG: glycerate 3-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate. The NOG architecture presented here considers promiscuous PKT activities previously described (Krüsemann et al., 2018). Multiple design of NOG exists and have been described (Bogorad et al., 2013). c) Reverse glyoxylate shunt for carbon conservation (Mainguet et al., 2013). In a wild-type *Escherichia coli*, conversion of succinate (SUC) to acetyl-CoA would go through two decarboxylation steps (red arrows): conversion of oxaloacetate (OAA) to PEP, and conversion of PYR to acetyl-CoA. Overall, this results in the yield of one acetyl-CoA per succinate (two out of four carbons, 50% yield). Instead, the use of gene deletions to prevent oxaloacetate (OAA) synthesis, in combination with heterologous production of Malate thiokinase (MTK), Malyl-CoA lyase (MCL), and ATP-citrate lyase (ACL), allowed to rewire TCA cycle intermediates towards the regeneration of OAA, thereby allowing microbial growth while generating two acetyl-CoA equivalents per succinate (four out of four carbons, 100% yield). However, for being operational the shunt required exogenous supplementation of malate, succinate and glucose. d) Carbon-conserving bypasses using pentoses. This panel summarizes key bypasses described in literature within the pentose network. The isomerase pathway (black lines) channels xylose into lower glycolysis *via* the pentose phosphate pathway. When producing the glutamate precursor 2-oxoglutarate (2OG), the isomerase pathway presents a maximum theoretical yield of 83% (five out of six carbons) due to decarboxylation of the C6 compound isocitrate (ICIT) within the TCA cycle (red arrow from CIT/ICIT to 2OG). A stoichiometrically advantageous route from xylose to 2OG is

represented by the Weimberg pathway, which results in a theoretical yield of 1 mol 2OG/mol xylose (5 out of 5 carbons conserved, 100% carbon yield). The network of xylose degradation allows to achieve carbon conservation also for the production of other target compounds, *e.g.* for derivatives of glycolaldehyde (as the C2 molecules glycolate or ethylene-glycol). A variant of the Weimberg pathway, the Dahms pathway, cleaves the intermediate 2-keto-3-deoxy-xylonate (2K3DX) into pyruvate (PYR) and glycolaldehyde. While the latter can be further converted into added-value products with a yield of 1 mol/mol xylose, the former can be used for gluconeogenesis. Synthetic variants for the generation of glycolaldehyde from xylose have been described in literature, such as the xylulose 1-phosphate (X1P) and the ribulose 1-phosphate (R1P) pathways. Both synthetic routes achieve glycolaldehyde production while generating the C3 compounds dihydroxyacetone phosphate (DHAP), which is more reduced than PYR and can be equally employed for gluconeogenesis. e) Carbon-positive bypasses within the pentose network. The four examples of bypasses in this panel result in stoichiometric gain by incorporating one carbon atom within the substrate backbone, generating either one C6 (*i.e.* F6P) or two C3 molecules (*i.e.* DHAP and GAP, G3P, or GAP and PYR) as products. The C1-compound requires reducing power for driving the assimilation step, either as NAD(*P*)H, or by harboring extra electrons within the molecule (*i.e.* formaldehyde). Abbreviations: RuMP, ribulose monophosphate; A3H6P, arabino-3-hexulose-6-phosphate; GED, Gnd-Entner-Doudoroff; XuMP, xylulose monophosphate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

avoiding the decarboxylation step from isocitrate to 2OG that would occur in the TCA cycle, which lowers the theoretically yield to 83% (5 out of 6 carbons conserved) (Radek et al., 2014). Therefore, the Weimberg pathway represents a bypass for stoichiometric gain and an alternative to the longer, carbon-inefficient route through PPP, lower glycolysis, and TCA cycle. The Weimberg pathway can be harnessed for the production of L-glutamate, which derives from 2OG (Bator et al., 2020). Nevertheless, by skipping substrate level phosphorylation of glycolysis, this metabolic route results in lower ATP availability for biomass formation, with consequent poor growth rates when growing on xylose, *e.g.* in *C. glutamicum* (Radek et al., 2014). Recently, the Weimberg pathway has been used to rescue a glutamate-auxotrophy in an itaconate-producing *E. coli*, where the isocitrate dehydrogenase gene (*icd*) has been deleted to increase availability of the precursor cis-aconitate (Lu et al., 2021).

Another way to generate carbon conservation within the pentose network is by cleaving the C5 substrate into C2 and C3 products. When focusing on the production of derivatives of acetyl-CoA, the use of PKTs for the cleavage of X5P into GAP and acetyl-P can be a valid option. Alternatively, and partially overlapping with the Weimberg route, the Dahms pathway branches from 2-keto-3-deoxyxylonate (common intermediate), which is then cleaved *via* an aldolase into pyruvate and glycolaldehyde (Fig. 3d). While the former is a key biomass precursor itself, the latter can be transformed into the C2 compound glyoxylate, which can also support biomass formation (Franden et al., 2018). Moreover, glycolaldehyde is precursor of the biotechnologically relevant monomers ethylene-glycol or glycolate. An *in silico* and *in vivo* comparison between isomerase, Weimberg, and Dahms pathways was performed in *Pseudomonas putida* catabolizing xylose (Bator et al., 2020), confirming different product yields depending on the compound of interest. With conversion of pyruvate into lactate, the Dahms pathway can be exploited also for high-yield synthesis of new polymers directly from xylose, such as poly(lactate-*co*-glycolate) (Choi et al., 2016). More generally, the Dahms pathway is optimal for synthesis of glycol-derived products, while still providing carbon for biomass formation.

Artificial variants to the pentose pathways have been created in *E. coli* to expand the potential of pentoses utilization (Fig. 3d). A synthetic xylose assimilation pathway was created converting the substrate into the intermediate xylulose-1-phosphate (X1P), which is then converted into glycolaldehyde and DHAP *via* aldolic cleavage (Cam et al., 2016). A similar architecture to the X1P pathway was designed in another synthetic route presenting ribulose 1-phosphate (R1P) as key intermediate (Pereira et al., 2016). As for the X1P pathway, R1P is cleaved to form glycolaldehyde and DHAP. Since R1P is an intermediate in the catabolism of various pentoses, the R1P pathway enables the consumption of many of them, including xylose and arabinose (Pereira et al., 2016).

2.1.2. Beyond carbon conservation: Carbon positive bypasses

When a surplus of electrons is present intracellularly, it can be used for driving carboxylation reactions. In literature, this has been already demonstrated, *e.g.* when inactivating electron consuming reactions in *E. coli* for the fermentation of glycerol to succinate (Yu et al., 2019b). In fact, by deleting the gene *pflB* encoding for pyruvate formate-lyase, production of the downstream products formate and ethanol was prevented anaerobically. Therefore, a higher NADH/NAD^+ could be used to revert the flux of the endogenous, gluconeogenic enzyme PEP carboxykinase (PEPCK), and support succinate formation *via* the reductive TCA cycle (Zhang et al., 2010). As alternative to PEPCK, a heterologous pyruvate carboxylase (PYC) was produced in *E. coli* to support carboxylation of pyruvate to OAA (Blankschien et al., 2010). Both examples demonstrate that, upon investment of an excess of reducing power, carbon assimilation reaction can be implemented within cell factories. We therefore describe this rationale for the design of carbon-positive bypasses.

Most of the examples of carbon-positive bypasses exist within the pentoses sub-network, and result in either one C6- or two C3-product molecules (Fig. 3e). The extra electrons required for these shunts are provided either in the form of NAD(*P*)H, or by using an already reduced form of CO_2 such as formate or formaldehyde. Different variants of carbon-positive bypasses have been proposed in literature and tested *in vivo*.

The first example we report was used to tackle redox balancing constraints during anaerobic fermentation processes. In the case of glucose fermentation into ethanol in yeast, reduction of DHAP to glycerol is a necessary redox sink for oxidizing excessive NADH. Overall, glycerol side-production negatively impacts ethanol yield. This was tackled by introducing phosphoribulokinase (PRK) and ribulose 1,5-bisphosphate carboxylase (Rubisco) in *S. cerevisiae*. Production of these two enzymes from the Calvin-Benson-Bassham (CBB) cycle resulted in a shunt converting ribulose 5-phosphate (R5P) to two glycerate 3-phosphate (3PG) molecules *via* CO_2 assimilation (Guadalupe-Medina et al., 2013) (Fig. 3e). Therefore, in this baker's yeast strain the excess of NADH could be harnessed for assimilating CO_2, overall increasing stoichiometric conversion of glucose into ethanol, while decreasing glycerol synthesis as by-product (Guadalupe-Medina et al., 2013; Papapetridis et al., 2018). This approach revealed to be useful for increasing the product yield and was extended to other cell factories like *E. coli* (Li et al., 2015; Tseng et al., 2018).

Another pathway for CO_2 (co-)assimilation, which was designed to consist fully of *E. coli* enzymes, is the GND-Entner-Doudoroff (GED) cycle (Satanowski et al., 2020). This CO_2 fixation cycle mirrors the structure of the canonical Calvin-Benson-Bassham (CBB) cycle. Although the GED pathway requires elevated CO_2 concentrations, it is more energetically efficient than the CBB cycle, as it consumes less ATP per pyruvate generated. The key reaction of this cycle is represented by the reverse reaction of 6-phosphogluconate (6PG) dehydrogenase (GND), which normally decarboxylates 6PG to ribulose 5-phosphate (Ru5P). The reductive carboxylation of Ru5P to 6PG using NADPH was achieved by harnessing this reverse reaction of GND (Satanowski et al., 2020). This synthetic shunt was demonstrated *in vivo* by overexpressing several native *E. coli* genes including *gnd*. Like the Rubisco shunt, GND allows to connect pentoses to C3 glycolytic intermediates (Fig. 3e) and could hence serve to obtain stochiometric gain in the conversion of pentoses (or hexoses) to C3, when extra electrons are available.

The use of CO_2 as a co-substrate for stochiometric gain can be cumbersome, especially due to its low-energy level, requiring additional reducing power for assimilation, as well as its relatively poor solubility in liquid media. To circumvent these limitations, reduced one-carbon feedstocks methanol and formate are promising (co-)substrates to realize stochiometric gains (Cotton et al., 2020; Stöckl et al., 2022). Methanol has a high degree of reduction ($\gamma = 6$ electrons per carbon), whereas formate has a lower one (2). The latter was shown to support the reverse activity of the PFL enzyme in *E. coli*, allowing condensation of acetate and formate to pyruvate (and therefore, growth) when the glyoxylate shunt was deleted (Δ*aceA*) (Zelcbuch et al., 2016). More recently, the use of formate as sink for anaerobic growth on glycerol was attempted *via* the reductive glycine pathway in *Clostridium pasteurianum* (Hong et al., 2021). This strategy can prevent the waste of electrons in the substrate when highly reduced glycerol is converted to more oxidized products, and could in principle be extended to other industrial microorganism for the stoichiometric gain on more-oxidized products (Claassens, 2021).

In addition to formate, methanol has been described in literature as supplementary feedstock for bypasses. To serve as carbon source, methanol is first oxidized into formaldehyde, a highly reactive (and toxic) compound which displays a similar degree of reduction to biomass (γ of 4 and 4.17 for formaldehyde and microbial biomass, respectively). Therefore, formaldehyde can be directly assimilated without the need of additional investment of reducing power (*e.g.* as from glycerol oxidation for formate). Two naturally existing pathways for methylotrophic growth -the ribulose monophosphate pathway (RuMP, typical of prokaryotes) and the xylulose monophosphate pathway (XuMP, present in methylotrophic yeasts) - offer stoichiometrically advantageous shunts from pentoses to glycolysis *via* formaldehyde assimilation (Fig. 3e). Both XuMP and RuMP have been engineered *in vivo* (Zhu et al., 2020), although the RuMP shunt has received more attention as it is energetically more efficient (Antoniewicz, 2019). The awareness towards this pathway led to the recent breakthrough of the full RuMP cycle established in *E. coli* (Chen et al., 2020). Of relevance for this review is the implementation of the C1-assimilation section of the cycle, the RuMP shunt. An elegant rewiring of *E. coli*'s metabolic network for the implementation of this bypass architecture was realized by screening formaldehyde assimilation modules through deletion strains. These were designed to display an incremental increase in their selective pressure, eventually allowing to support the synthesis of a high fraction (> 70%) of biomass precursors through the RuMP shunt activity condensing Ru5P (obtained from xylose) and formaldehyde (He et al., 2018). Another recent work demonstrated restoration of full growth on xylose and methanol *via* the RuMP shunt in a "synthetic methanol auxotroph" *E. coli* strain by combining rational design with adaptive laboratory evolution (Chen et al., 2018). Moreover, in the latter work, authors could demonstrate synthesis of ethanol and 1-butanol through this bypass. Therefore, multiple options for carbon-positive bypasses exist within the pentose network which can be used for increasing stoichiometric gain of carbon compounds.

2.1.3. *Bypasses for stoichiometric advantages in secondary metabolism – The example of isoprenoid biosynthesis*

The design principle of stoichiometric gain can be extended also to secondary metabolism. As an example, we focus on the synthesis of isoprenoids, known as attractive biotechnological products. This class of molecules is composed by about 60,000 compounds, displaying a wide range of applications within the bio-based economy, including pharmaceuticals, flavors and fragrances, commodity chemicals, and biofuels (Li et al., 2020). All isoprenoids originate from the two isomeric universal precursors, the C5-molecules isopentenyl-pyrophosphate (IPP) and dimethylallyl-pyrophosphate (DMAPP). Streamlining cell factories' metabolic flux to support high IPP and DMAPP productivities is one of the key metabolic targets for the synthesis of a plethora of isoprenoid molecules (Daletos et al., 2020). Two natural pathways synthesize IPP and DMAPP: the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, and the mevalonate (MVA) pathway. With few exceptions, these two metabolic routes are phylogenetically distinct, with the MEP pathway present in bacteria, whereas the MVA is found in archaea and eukaryotes. Since these two pathways branch from different nodes of metabolism, they constitute natural bypasses: the MEP pathway starts with condensation of GAP and pyruvate to generate 1-deoxy-D-xylulose 5-phosphate (DXP) and CO_2, whereas the MVA one with the condensation of acetoacetyl-CoA and acetyl-CoA to generate hydroxymethylglutaryl-CoA (HMG-CoA), which is further converted to MVA and decarboxylated to IPP through additional four enzymatic reactions.

The MEP pathway is stoichiometrically superior to the MVA counterpart. Theoretically, one glucose is necessary for generating one IPP equivalent *via* the former. Conversely, 1.5 glucose are needed to generate three acetyl-CoAs, which are condensed through the MVA pathway, generating one IPP equivalent (Fig. 4). For this reason, functional substitution of the native MVA with a bacterial MEP pathway has long been a goal for increasing the theoretical isoprenoid yield in the eukaryotic workhorse *S. cerevisiae*, although it has not been achieved yet (Carlsen et al., 2013; Kirby et al., 2016; Partow et al., 2012). In fact, an inherent challenge characterizes the last two step of the MEP pathway, catalyzed by ISPG and ISPH. These two enzymes contain [4Fe-4S] clusters, whose assembly machinery is difficult to produce in yeast's cytosol by means of heterologous gene expression (Kirby et al., 2016).

An alternative way to achieve stoichiometric gain in isoprenoid biosynthesis would be the direct conversion of C5 substrates into the key C5-metabolites IPP and DMAPP. However, no enzymes were known to connect pentose sugars to these precursors. To realize this conversion, promiscuous enzyme activities were exploited. This approach is possible especially in secondary metabolism, where enzymes are less specialized (Bar-Even and Tawfik, 2013). To identify promiscuous activities connecting pentose sugars with the MEP pathway, a growth-coupled selection strategy was designed. For this purpose, a gene encoding the first enzyme of the pathway (*dxs*) was deleted in *E. coli* to interrupt the connection between the glycolysis and IPP/DMAPP biosynthesis. Then, the engineered *E. coli* strain was transformed with the genes encoding the lower part of the MVA pathway and supplemented with MVA to restore growth. This strain was then used as platform for *in vivo* enzyme screening to find new enzyme activities to supply the upper MEP pathway from the pentose phosphate pathway. Initially, this selection strain was supplemented with sufficient MVA to allow growth. However, by lowering the concentration of supplemented MVA, spontaneous mutants which could synthesize DXP were capable of restoring growth. Two enzymes demonstrated to support the conversion from Ru5P to DXP and, as proof-of-principle, this bypass - named nDXP (Fig. 4) - was further confirmed to sustain biosynthesis of the biofuel bisabolene in *E. coli* (Kirby et al., 2015).

In summary, stoichiometrically advantageous bypasses can be designed in both central and secondary metabolism. We have observed that their implementation can not only decrease carbon loss but obtain net carbon gain by assimilating C1 moieties (CO_2 or formaldehyde) within the carbon backbone of central metabolism intermediates. However, in the case of CO_2 fixation, investment of reducing equivalent must be accounted, as its null degree of reduction ($\gamma = 0$) leaves this molecule non-reactive.

2.2. *Avoiding kinetic limitations and unfavorable promiscuities*

Each metabolic route contains one or more enzymes that exert some degree of control over the pathway activity. For example, an enzyme can be subjected to feedback inhibition from the product, a characteristic that generally has a high influence over the pathway's downstream metabolite concentrations (Fell, 1998). Alternatively, some other enzymes might present poor kinetic parameters (K_M or k_{cat}), which can constitute a rate-limiting step. These drawbacks can be avoided, *e.g.*, by random or targeted protein engineering for removal of feedback

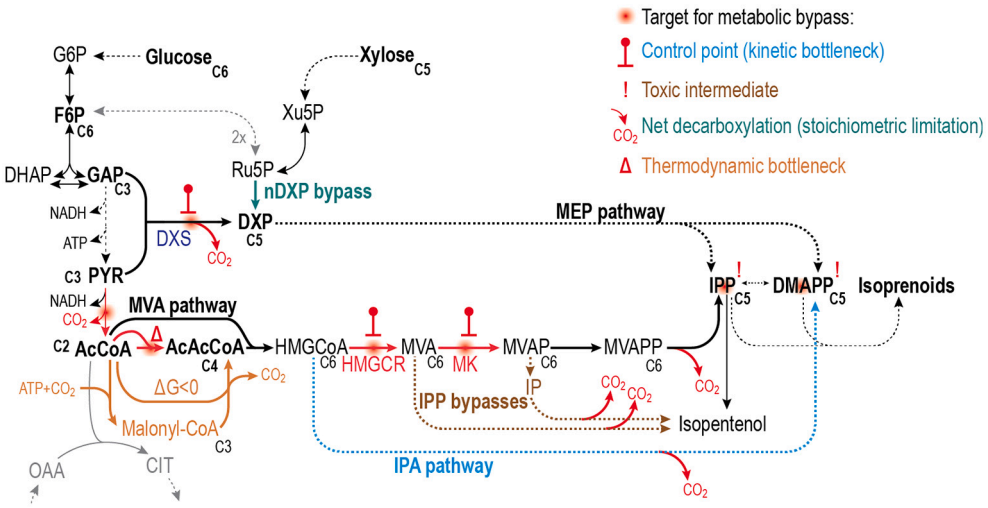

Fig. 4. Bypasses within the isoprenoid biosynthetic network. Isoprenoid biosynthesis well summarizes a network where multiple design principles can be implemented at once. Bottlenecks are highlighted with a red shadow, and the type of design principle for their circumvention is also presented with a cartoon. The use of bypasses expands the topology within this subnetwork of secondary metabolism. The MEP pathway, typical of bacteria, is stoichiometrically superior to the eukaryotic MVA pathway because of reduced carbon loss. The latter is characterized by two decarboxylation events: the first happens three times from pyruvate (PYR, C3) to acetyl-CoA (C2), and the second occurs once from mevalonate diphosphate (MVAPP, C6) to isopentenyl diphosphate (IPP, C5). Instead, the MEP pathway is characterized by only one decarboxylation step (condensation of GAP and PYR, both C3) catalyzed by deoxyxylulose 5-phosphate synthase (DXS), which generates 1-deoxy-D-xylulose 5-phosphate (DXP, C5). DXS is also an important control point of the pathway. Therefore, these two stoichiometric and kinetic limitations justified the implementation of the nDXP bypass, consisting in a shunt from pentoses to DXP. The MVA pathway presents additional limitations, such as the thermodynamic bottleneck for the condensation of two acetyl-CoA into acetoacetyl-CoA (AcAcCoA). The design of a malonyl-CoA bypass (at the expense of one ATP) can circumvent this thermodynamic barrier and allow the generation of AcAcCoA with an overall $\Delta_r G'^{m} < 0$. The kinetic limitations of two enzymes downstream of AcAcCoA, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and mevalonate kinase (MK) can be circumvented by exploiting the isoprenoid alcohol (IPA) pathway. Lastly, the toxic nature of IPP and dimethylallyl diphosphate (DMAPP) was circumvented by designing two bypasses to produce isopentenol, which converted MVA or MVAP directly to the product molecule. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibition, or *via* gene overexpression, respectively (Davy et al., 2017). In this section, we reason that an additional option to overcome kinetically challenging enzymatic steps is to completely circumvent them through bypasses.

The PEP-pyruvate-oxaloacetate node is a metabolic hub at the intersection of glycolysis and the TCA cycle, and shows a high enzymatic variation among the three domains of life (Koendjbiharie et al., 2020; Sauer and Eikmanns, 2005). In *E. coli*, PEP carboxylase (PEPC) is the natural enzyme involved in the carboxylation of PEP into OAA. However, this architecture can be kinetically inadequate when fermenting glycerol to succinate. In fact, in this bioproduction process a C3 molecule from lower glycolysis must be carboxylated into a C4 intermediate of the TCA cycle, which then gets reduced *via* a reverse TCA cycle into succinate (Fig. 5a). The anaerobic route for glycerol catabolism involves a glycerol dehydrogenase (GLDA) and a dihydroxyacetone kinase (DHAKLM). The latter step catalyzes the transfer of a phosphate group from PEP to dihydroxyacetone (DHA), generating DHAP and pyruvate as products (Fig. 5a). This step decreases the intracellular pool of PEP, which can be restored from pyruvate by investing two ATP equivalents. However, the kinetic parameters around the PEP node are unfavorable for its carboxylation, as this molecule has a higher affinity towards DHAKLM than PEPC, which present K_M values for PEP of 0.045 and 0.25 mM, respectively (Blankschien et al., 2010). Another endogenous enzyme, PEP carboxykinase (PEPCK) could in principle support carboxylation but has a very low affinity for bicarbonate (K_M of 13.0 mM for HCO_3^-). Similarly, the native malic enzyme (MAE) displays a much higher affinity towards the decarboxylative direction of the reaction (K_M of 0.26 and 16.0 mM for malate and pyruvate, respectively). A solution to this problem was presented by producing the pyruvate carboxylase (PYC) enzyme from *Lactococcus lactis* in *E. coli* (Blankschien et al., 2010), which has a k_{cat} of 192 s^{-1} and a K_M towards pyruvate of 3.1 mM (Choi et al., 2017). Therefore, use of PYC allowed effective production of succinate out of the pyruvate node, thereby circumventing the competition between DHAKLM and the carboxylation enzymes at the PEP node (Blankschien et al., 2010). In principle, *pyc* does not require any stronger expression than *pepc* for the bypass to be effective. Inactivation of the latter combined to physiological levels of the PYC enzyme presenting favorable kinetics should be enough to drive the reaction in the desired direction. Finally, it is worth noting that the bypass through PYC requires the investment of only one ATP, whereas the route *via* PEPC would require the prior conversion of pyruvate into PEP, which costs two ATP equivalents. Therefore, the use of PYC is also advantageous in terms of ATP cost.

Another competition exists between two enzymes at the PEP node: the sugar-transporting PEP:carbohydrate phosphotransferase system (PTS) and the 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthase (Fig. 5b). The latter catalyzes the first step of the shikimate pathway, a biosynthetic route of aromatic compounds, by condensing PEP with E4P to form DAHP (Fig. 5b). However, when growing on glucose this enzyme directly competes with PTS for PEP, as it transfers PEP's phosphate group to glucose, generating G6P and pyruvate (Fig. 5b). Therefore, PTS activity diminishes the intracellular PEP pool available for DAHP synthase, with consequent decrease on the enzyme's reaction rate -$K_{M,\ PEP}$ of 35 μM for the DAHP synthase isoenzyme AROG (Jianfeng et al., 2004)- and overall volumetric productivity of aromatic compounds. To tackle this bottleneck, the Frost group published two consecutive papers which describe the redesign of the shikimate entry point, and the following improvement of its catalytic rates for improved pathway flux. First, the ED pathway enzyme 2-keto-3-deoxy-6-phosphogalactonate (KDPGAL) aldolase was modified in its activity to serve as replacement of DAHP synthase. This enzyme, which normally cleaves KDPGAL into GAP and pyruvate, was modified for increasing its specificity towards E4P, thereby allowing condensation of pyruvate and E4P, resulting in DAHP generation. For this purpose, the KDPGAL from *Pseudomonas cepacian* was cloned in *E. coli*, as this enzyme already proved to catalyze condensation of pyruvate with E4P (Ran et al., 2004). After demonstrating the condensation *in vitro*, directed evolution was combined with growth-based selection to demonstrate pyruvate and E4P condensation also *in vivo* in the *E. coli* strain CB734, which lacks all DAHP synthase isoenzymes. Therefore, authors could demonstrate the ability of the engineered *E. coli* host to restore growth without any aromatic supplementation (Ran et al., 2004). In a follow-up

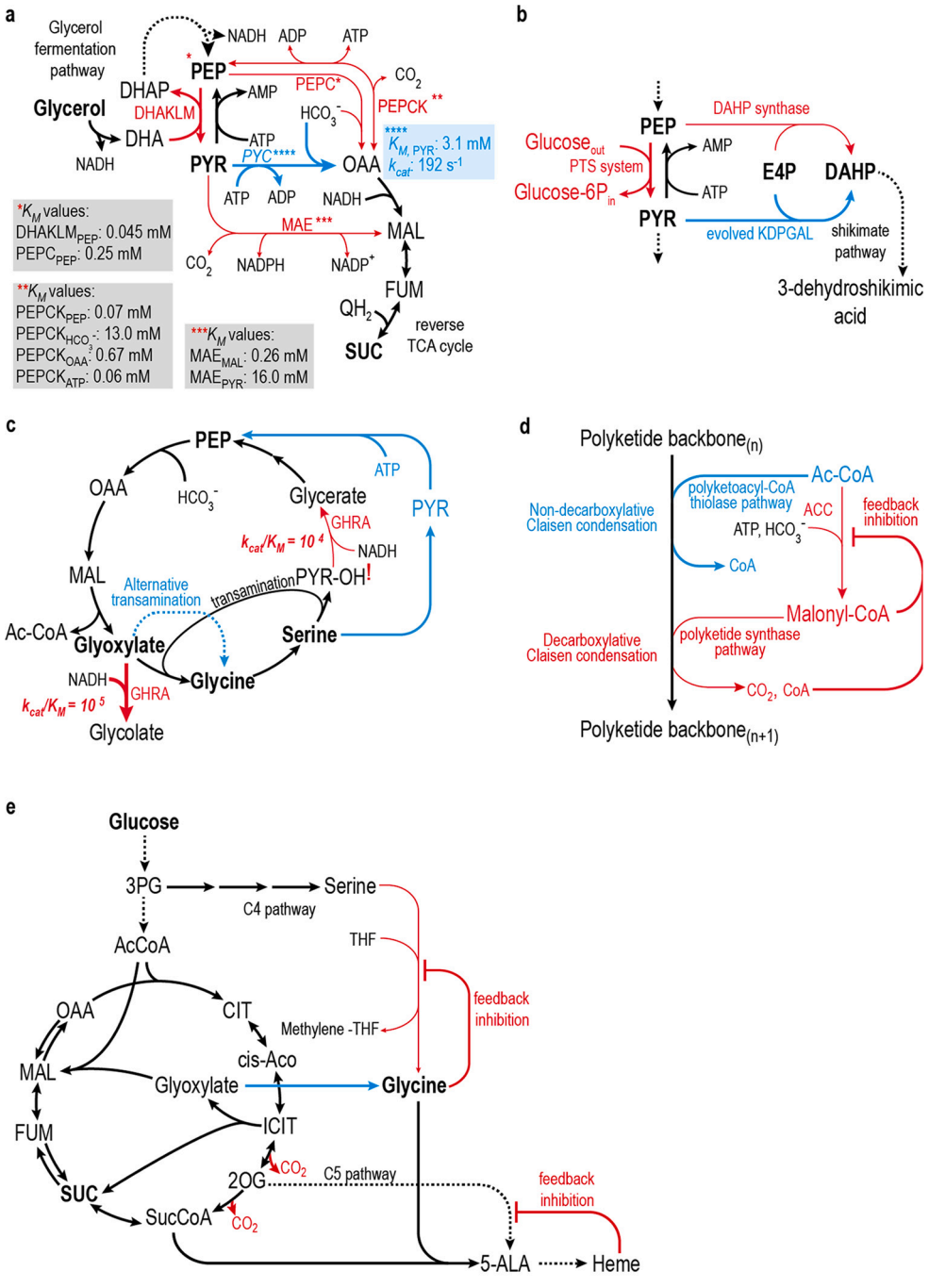

Fig. 5. Avoiding kinetic limitations and unfavorable promiscuities in *E. coli*. a) Engineering a heterologous pyruvate carboxylase bypass for glycerol fermentation to succinate. The native architecture around the PEP node results in a competition between DHAKLM (involved in fermentative glycerol catabolism) and the carboxylation enzymes at the PEP node (PEPC and PEPCK). Pyruvate (PYR) can in principle be carboxylated to malate, but the kinetics of this reaction are challenging for HCO_3^-. Introduction of a heterologous pyruvate carboxylase (PYC, cyan arrow) allows effective carboxylation to OAA, thereby bypassing the competition at the PEP node (Blankschien et al., 2010). b) Redesign of the entry point of the shikimate pathway, which tackles the limitation of PEP consumption by the PTS system for glucose uptake (red arrows). By evolving KDPGAL to accept PYR for the condensation with E4P (cyan arrows), competition between PTS and DHAP synthase was released (Ran et al., 2004; Ran and Frost, 2007). c) Introduction of a serine cycle in *E. coli* requires the implementation of bypasses to circumvent GHRA, which displays a higher activity for a glyoxylate-consuming reaction (thick red arrow), which inevitably depletes the cycle (Yu and Liao, 2018). The bypass is depicted by cyan arrows and required the use of an alternative transamination for converting glyoxylate to glycine. The intermediate hydroxypyruvate (PYR-OH) is also a toxic compound (highlighted by a red exclamation mark), and its bypass is also discussed in the section of the manuscript about circumventing toxic intermediates. d) Bypass for improving the elongation phase during polyketide biosynthesis. Acetyl-CoA carboxylase (ACC) is subjected to feedback inhibition from malonyl-CoA and CoA, which entangles polyketide backbone elongation. Instead, use of the polyketoacyl-CoA thiolases pathway (cyan arrows) bypasses this kinetic limitation (Tan et al., 2020). e) Alternative glycine biosynthesis to produce 5-aminolevulinic acid (5-ALA). 5-ALA can be generated in a one-step reaction from glycine and succinyl-CoA. Canonical generation of the precursor glycine occurs through the serine pathway (C4 pathway). To avoid this route, glycine synthesis was redesigned through a retrosynthetic approach, and generated directly from glyoxylate (Ren et al., 2018). Thin red arrows: kinetically limited reaction. Thick red arrows: competitive reactions for a key intermediate. Cyan arrows: bypasses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

study, authors performed directed evolution on three KDPGAL aldolase orthologs (from *E. coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*), followed by DNA shuffling of the evolved sequences. Eventually, the kinetic parameters of the best evolved enzyme improved several folds, with the $K_{M,\ E4P}$ decreasing from 570 to 49 μM, and the k_{cat} increasing from 0.94 to 4.9 s^{-1}, compared to the starting *E. coli* WT DHAP synthase. Overall, these improvements determined a 60-fold increase in the k_{cat}/K_M of the enzyme. Consequently, authors could eventually harness the advantage of this modified entry point in the shikimate pathway for demonstrating final yield of 3-dehydroshikimic acid from glucose of 18% mol/mol (Ran and Frost, 2007).

The serine cycle is a relevant C1-assimilating pathway, which can serve to aerobically assimilate diverse C1-substrates including methanol and formate. This metabolic route relies on carboxylation steps that can occur at ambient CO_2. One is through phosphoenolpyruvate (PEP) carboxylase, one of the best performing carbon capturing enzymes (Cotton et al., 2018). This enzyme assimilates HCO_3^-, generating OAA from PEP. The other C1 assimilation step is catalyzed by serine

hydroxymethyl transferase, which transfers one carbon from 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) to glycine, generating serine. 5,10-CH_2-THF can be derived from either formate or methanol as feedstocks. The serine cycle directly generates acetyl-CoA as product, thereby offering the chance to synthesize acetyl-CoA derivative compounds while avoiding any carbon-inefficient decarboxylation from pyruvate. A modified serine cycle, displaying some bypasses to the natural variant, was established in *E. coli* to support the conversion of methanol (or formate) and CO_2, together with xylose, into C2 compounds. (Yu and Liao, 2018). To adapt and implement the structure of the serine cycle from *Methylobacterium extorquens AM1*, this work firstly characterized the endogenous serine metabolism in *E. coli*. Regarding the bypassing of a kinetic bottleneck, particular attention must be given to the steps involving the conversion of glyoxylate to PEP (Fig. 5c). These start with the amination of glyoxylate to glycine, followed by the assimilation of the C1 moiety from 5,10-CH_2-THF to generate serine. Then, the generated serine is used as an amino donor for the reaction from glyoxylate to glycine, producing hydroxypyruvate. In the native serine cycle in *M. extroquens*, the latter molecule is further reduced to glycerate, a reaction that in *E. coli* is catalyzed by the protein hydroxypyruvate reductase (GHRA). Then, a two-step conversion using glycerate kinase and enolase converts glycerate to PEP. In respect to the kinetic parameters for hydroxypyruvate, *E. coli's* GHRA presents better kinetic properties (lower K_M and higher V_{MAX}) for the (unwanted) reduction of glyoxylate to glycolate (Nuñez et al., 2001). This results in a 10-fold higher catalytic efficiency of the enzyme with glyoxylate compared to hydroxypyruvate, with a k_{cat}/K_M of 10^5 and 10^4, respectively (Fig. 5c) (Nuñez et al., 2001). Therefore, reduction of glyoxylate is a reaction that drains the intracellular glyoxylate pool available for the transamination required by the cycle. To prevent this drawback, the cycle was redesigned circumventing GHRA. Authors implemented a bypass to connect serine to PEP involving pyruvate as intermediate (Fig. 5c). Moreover, to generate glycine from glyoxylate, they implemented an alternative transamination reaction, which involved the enzyme alanine-glyoxylate transaminase (Fig. 5c) (Yu and Liao, 2018). The *E. coli* strain generated could therefore bypass GHRA, supporting *in vivo* C1 assimilation (formate and methanol) when using xylose as co-substrate (Yu and Liao, 2018). Eventually, as proof-of-principle ethanol production was also demonstrated through this cultivation setup (Yu and Liao, 2018).

Another kinetic obstacle, the first step of the MEP pathway, has been well characterized in secondary metabolism. In fact, although stoichiometrically superior, the MEP pathway is inherently regulated by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). This is the first enzyme of the pathway and acts as crucial kinetic control point (Banerjee and Sharkey, 2014; Volke et al., 2019). Two cases reported in literature managed to channel flux from pentoses directly to DXP, thereby avoiding the first committed step of the canonical MEP pathway in *E. coli*. The first example is the nDXP bypass, which was also presented previously as a shunt for carbon conservation (Kirby et al., 2015) (Fig. 4). The second one consists in the design of a novel entry point to DXP through the promiscuous activity of F6P aldolase (King et al., 2017). Also in this case, the justification was to avoid DXS by creating a *de novo* bypass to DXP starting from the catabolism of pentoses. However, here DXP generation was obtained by condensation of the C2 molecule glycolaldehyde and the C3 molecule hydroxyacetone. Similarly, the bypass for isopentenol production allowed to avoid carbon flux through mevalonate kinase, another kinetically limiting enzyme of the MVA pathway (Kang et al., 2016) (Fig. 4). The same narrative for avoiding kinetic limitations can be applied when studying the synthesis of the universal isoprenoid precursors IPP and DMAPP. In bacteria, their synthesis *via* the MEP pathway is tightly regulated at the transcriptional and post-transcriptional level (Banerjee and Sharkey, 2014). This motivated the expression of an heterologous MVA pathway in *E. coli* on top of the endogenous MEP pathway (Martin et al., 2003). Complete functional replacement of the MEP pathway with the MVA pathway was later characterized in another prokaryotic cell factory, *Rhodobacter sphaeroides* (Orsi et al., 2020b), eventually demonstrating its benefit for feasible isoprenoid production also during non-growing conditions (Orsi et al., 2020c). The isoprenoid alcohol pathway (IPA, Fig. 4) is a synthetic route built for bypassing an endogenous complex regulation at the level of HMG-CoA reductase (Kizer et al., 2008). This pathway shares the first 3 committed steps with the MVA pathway, and successfully generates IPP and DMAPP through a new set of reactions generated by combining in a 'mix and match' both endogenous and heterologous enzymes (Clomburg et al., 2019).

Polyketides are another class of natural secondary products with high potential as pharmaceuticals (Staunton and Weissman, 2001). Their synthesis can be divided in a starting phase (usually involving acetyl-CoA), followed by an elongation and a termination phase. The elongation phase usually relies on malonyl-CoA as extender unit, which is incorporated into the polyketide backbone through a decarboxylative Claisen condensation catalyzed by polyketide synthase (Fig. 5d). However, relying on malonyl-CoA has several disadvantages, including kinetic implications. Firstly, malonyl-CoA acts as allosteric inhibitor on the producing enzyme ACC (Brownsey et al., 2006). Second, this molecule is a key precursor for fatty acids biosynthesis, an essential metabolic route which competes with polyketide biosynthesis for the availability of malonyl-CoA. A third (stoichiometric) drawback is that generation of malonyl-CoA from acetyl-CoA requires the investment of one ATP, with consequent energetic inefficiency. Therefore, these three disadvantages motivated the Gonzalez group to find an alternative to bypass the need of malonyl-CoA as extender unit. In a recent work (Tan et al., 2020), the authors redesigned the polyketide biosynthesis in *E. coli* to rely on acetyl-CoA instead of malonyl-CoA as extender unit. In a nutshell, the authors mined the thiolase superfamily looking for candidates capable of catalyzing a non-decarboxylative Claisen condensation, which therefore could use acetyl-CoA as substrate. Therefore, they identified non-decarboxylative polyketoacyl-CoA thiolases as candidates and tested them based on their catalytic properties. An advantage of this approach is that, due to the higher intracellular flux towards acetyl-CoA over malonyl-CoA (Krivoruchko et al., 2015), a higher pool of the extender acetyl-CoA unit could in principle be used in the new polyketide biosynthetic route. As proof-of-principle, the synthesis of triacetic acid lactone, orsellinic acid, and orcinol was tested *in vivo* using acetyl-CoA as extender unit (Tan et al., 2020).

Another example for the design of a bypass of a kinetic bottleneck involves a new-to-nature shunt for the synthesis of glycine as precursor of 5-aminolevulinic acid (5-ALA). In *E. coli*, synthesis of ALA can occur either *via* a 'C5' or a 'C4' route, depending on the carbon skeleton of the precursor. In both cases, kinetic limitations challenge the efficient production of this compound (Fig. 5e). In fact, the end product heme (downstream of 5-ALA) exerts feedback inhibition on the enzymes of the 'C5 pathway' Glutamyl-tRNA reductase (HEMAS) and Glutamate-tRNA ligase (GLTX). The 'C4 pathway' is shorter and needs only two precursors, succinyl-CoA and glycine. However, generation of glycine from serine *via* the 'C4 pathway' is subjected to complex regulation at the level of the enzyme serine hydroxymethyltransferase (SHMT), which is also subjected to feedback-inhibition by glycine and other metabolites. Therefore, supply of glycine is the limiting factor for high-yield and -productivity of 5-ALA. By redesigning glycine biosynthesis directly from glyoxylate through transamination, it was possible to bypass the complex regulation of the 'C4 pathway' and support 5-ALA biosynthesis at high titers (Ren et al., 2018) (Fig. 5e).

The wide variety of examples for bypassing kinetic limitations demonstrates how this strategy is a valid alternative for the circumvention of kinetic bottlenecks. Moreover, it allows metabolic engineers to tackle limitations of, *e.g.*, rate-limiting enzymes without the need of additional expertise from other related disciplines, such as rational protein engineering.

2.3. Increasing thermodynamic driving force

Pathways with a similar metabolic function can display different thermodynamic profiles. This was previously reported at the level of glycolysis, where the Entner–Doudoroff (ED) pathway resulted to have a higher overall difference in Gibbs energy (Δ_rG') in its thermodynamic profile compared to the EMP pathway (Δ_rG' of -165 *versus* -100 kJ/mol, respectively) (Flamholz et al., 2013). This higher thermodynamic drive of the ED pathway is however associated to the harvest of less ATP, whereas the constraint of the EMP pathway reflects in roughly three- to five-fold higher fraction of the proteome invested for achieving the same flux. The recent activation of silent glycolytic bypasses of the EMP pathway in *E. coli* (Iacometti et al., 2022) confirms the feasibility of generating bypasses by following thermodynamic design principles, which is the topic of this section. As we will see in more detail in Section 2.6, choice of using GAPN as enzyme to catalyze NADPH generation also follows a thermodynamic principle. By sacrificing the harvesting of one ATP while generating NADPH, the Gibbs free energy of the conversion of GAP into 3PG is kept at -48.0 ± 1.1 [kJ/mol], which guarantees feasible production of this cofactor under physiological conditions.

When looking at thermodynamic limitations, one type of metabolic bottleneck that can be effectively overtaken by using bypasses is represented by reactions whose reversibility is unfeasible under physiological conditions. In particular, by transferring the energy release of ATP hydrolysis to endergonic reactions, it was possible to reverse the flux of some otherwise challenging conversions with a $\Delta_rG'^m >> 0$, with $\Delta_rG'^m$ defined as the Gibbs energy of the reaction under physiological conditions: 1 mM of each reactant, pH of 7.5, and ionic strength of 0.25 M (Flamholz et al., 2012). The first example of this category is represented by the malonyl-CoA bypass for driving the condensation of two acetyl-CoA into acetoacetyl-CoA. The latter molecule is precursor of several biosynthetic routes, *e.g.* poly(3-hydroxybutyrate) (PHB) and the mevalonate pathway for isoprenoid production (Figs. 4, 6a). Acetoacetyl-CoA is synthesized *via* thioester-dependent Claisen condensation of two molecules of acetyl-CoA, with subsequent release of free CoA, through the activity of acetyl-CoA *C*-acetyltransferase (EC 2.3.1.9). The reaction catalyzed by this enzyme presents a positive $\Delta_rG'^m$ of 26.1 ± 1.7 [kJ/mol]. Metabolic control analysis on the PHB biosynthetic route revealed a high control coefficient for the intracellular [acetyl-CoA]/[CoA] ratio (Van Wegen et al., 2001), which regulates the first step of the pathway responsible of acetoacetyl-CoA synthesis. Therefore, a high intracellular acetyl-CoA pool is required for the reaction to occur in the forward direction. An enzyme belonging to the acetoacetyl-CoA synthase superfamily, NPHT7, was proven to condensate acetyl-CoA and malonyl-CoA, forming acetoacetyl-CoA, CoA and CO_2 (Okamura et al., 2010). Its activity has been combined with the endogenous acetyl-CoA carboxylase, which carboxylates acetyl-CoA to malonyl-CoA by consuming one ATP, to generate a thermodynamically favorable bypass (Lan and Liao, 2012). In fact, the two reactions present a $\Delta_rG'^m$ of -9.1 ± 2.9 [kJ/mol] and -8.8 ± 6.3 [kJ/mol] for the carboxylase and the synthase, respectively. CO_2 functions as catalyst, since no net carbon is assimilated in the two reactions. This bypass exemplifies the advantage of investing one ATP for bypassing a thermodynamic barrier, while concurrently avoiding the kinetic limitation exerted by acetyl-CoA *C*-acetyltransferase on the downstream production pathways. Because of these two aspects, this shunt is indicated as an attractive engineering step to implement in autotrophs, such as the cyanobacterium *Synechococcus elongatus* (Lan and Liao, 2012; Lee et al., 2020), which generally present a low acetyl-CoA pool, expressed as a low [acetyl-CoA]/[CoA] ratio.

The same group tackled the challenge of reversibility within the glyoxylate shunt with the same strategy. This pathway was already presented as a synthetic bypass designed for carbon conservation (Mainguet et al., 2013). Two of the cycle reactions were thermodynamically unfeasible in the metabolic context of *E. coli*: the reverse activities of malate synthase ($\Delta_rG'^m$ = 38.7 ± 4.1 kJ/mol), and citrate synthase ($\Delta_rG'^m$ = 38.8 ± 0.9 kJ/mol). To circumvent these bottlenecks, thermodynamic principles combined with heterologous enzyme expression were employed to engineer the pathway, utilizing ATP to drive the key steps (Fig. 6b). More precisely, the irreversible reaction of malate synthase was tackled by harnessing two heterologous reactions belonging to the serine cycle. The first one was an ATP-dependent malate-thiokinase from *Methylococcus capsulatus* ($\Delta_rG'^m$ = -7.2 ± 7.0 kJ/mol), which activated malate to malyl-CoA by investing one ATP. Then, malyl-CoA was further converted into glyoxylate and acetyl-CoA *via* a malyl-CoA lyase from *Rhodobacter sphaeroides* ($\Delta_rG'^m$ = -0.9 ± 5.7 kJ/mol). To revert the reaction catalyzed by citrate synthase, a heterologous ATP-dependent citrate lyase from *Chlorobium tepidum* was cloned,

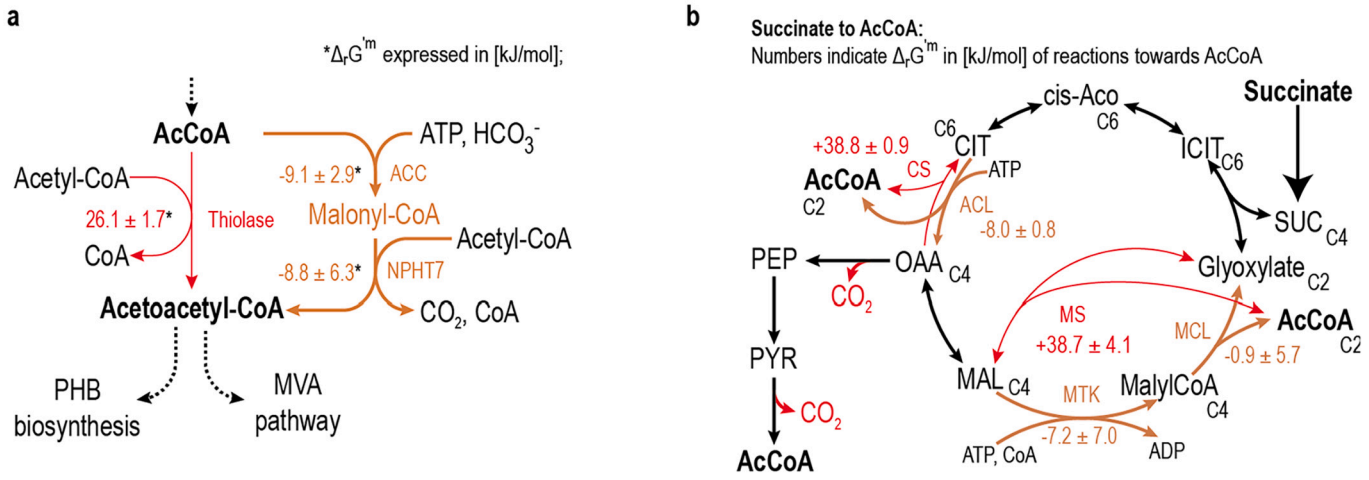

Fig. 6. Example of bypasses for increasing the thermodynamic driving force (orange arrows). A) Engineered malonyl-CoA bypass in cyanobacteria. The enzyme acetoacetyl-CoA thiolase (red arrows) catalyzes the Claisen condensation of two acetyl-CoA. However, this reaction presents a $\Delta_rG'^m > 0$, making it challenging to proceed in the condensation direction, unless a high intracellular [acetyl-CoA]/[CoA] is guaranteed. To prevent this bottleneck, the enzyme acetyl-CoA carboxylase (ACC) can carboxylate acetyl-CoA to malonyl-CoA by investing one ATP. Then, the enzyme NPHT7 can catalyze a decarboxylating condensing reaction of malonyl-CoA with acetyl-CoA to generate acetoacetyl-CoA. Both reactions characterizing this bypass (orange arrows) display a $\Delta_rG'^m < 0$. b) Heterologous production of the ATP-consuming enzymes malate thiokinase (MTK, orange arrow) and ATP-citrate lyase (ACL, orange arrow), in combination with malyl-CoA lyase (MCL, orange arrow), allowed to reverse the glyoxylate shunt in *E. coli* by circumventing the irreversible reactions of malate synthase (MS) and citrate synthase (CS), both in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which catalyzed the conversion of citrate to OAA and acetyl-CoA by investing 1 ATP molecule ($\Delta_r G'^m = -8.0 \pm 0.8$ kJ/mol). Eventually, this study resulted in a proof-of-principle for implementing a bypass for stoichiometric gain, where two challenging, irreversible enzymatic steps were elegantly circumvented by investing ATP.

2.4. Circumventing cytotoxic intermediates

Often, enzyme levels are increased in metabolic engineering to enhance carbon flux through a biosynthetic pathway. However, this strategy can lead to a series of unfavorable factors, such as metabolic burden from protein production, and accumulation of intermediates that can have an inhibitory or cytotoxic effect (Kizer et al., 2008). To tackle the latter challenge, many 'detoxification' engineering efforts have been proposed, *e.g.* by quickly removing inhibitory intermediates from the pathway by overexpressing genes of consuming reactions (Withers et al., 2007; Zhu et al., 2002), or by establishing dynamic expression systems based on the response to the specific metabolite concentrations (Dahl et al., 2013). However, an emerging approach involves rewiring the carbon fluxes for completely bypassing the synthesis of a toxic intermediate. This optimization principle characterizes this fourth section of the review.

1,2-propanediol (1,2-PDO) is widely used in the chemical industry, and different options for its bio-based production are being explored. The set of feedstocks supporting the synthesis of this compound is vast, involving multiple bioproduction pathways, and this has been recently reviewed in detail (Tao et al., 2021). When growing on glucose, 1,2-PDO biosynthesis is driven by the methylglyoxal route. This branches from glycolysis *via* the enzyme methylglyoxal synthase (MGSA), which starts by converting DHAP to methylglyoxal (Fig. 7). The toxic nature of methylglyoxal is a hurdle that justified the testing of alternative routes to 1,2-PDO. In particular, building on the discovery that anaerobic lactate-degraders generate 1,2-PDO (Oude Elferink et al., 2001), it was possible to design and test a new pathway in *E. coli* for the conversion of lactate into 1,2-PDO *via* a retrosynthetic approach (Niu and Guo, 2015). Then, this new artificial route was successfully tested for the synthesis at high titers of 1,2-PDO directly from glucose, while completely circumventing methylglyoxal accumulation (Fig. 7) (Niu et al., 2019).

The natural variant of the C1-assimilating serine cycle pathway generates hydroxypyruvate (Fig. 5c). This intermediate is highly

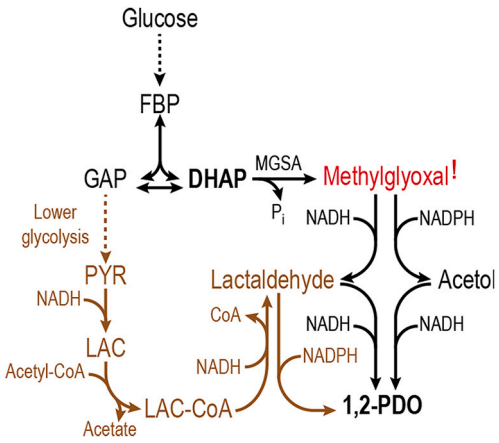

Fig. 7. Example of bypass for a toxic intermediate (methylglyoxal, red with exclamation mark) by redesigning 1,2-propanediol (1,2-PDO) biosynthesis in *E. coli*. Instead of relying on the dephosphorylation of DHAP to methylglyoxal, it was possible to redirect the carbon flux (brown arrows) to lower glycolysis, and subsequently convert pyruvate (PYR) to lactate (LAC). LAC was then activated to LAC-CoA and further reduced to lactaldehyde, and finally to 1,2-PDO, through a synthetic pathway (Niu et al., 2019; Niu and Guo, 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reactive and can trigger several detrimental reactions for cellular homeostasis (de Lorenzo et al., 2015). As we saw in the previous Section 2.2, the strategy of adapting the serine cycle to bypass hydroxypyruvate was originally justified by kinetic limitations within the endogenous *E. coli*'s serine metabolism (Yu and Liao, 2018). However, the design proposed by the authors also allowed to avoid the generation of this toxic intermediate (Fig. 5c).

Another example of cytotoxic bottlenecks is found in the isoprenoid pathways, in which IPP has a toxic nature (George et al., 2018). In the case of isopentenol production, endogenous metabolism requires accumulation of IPP, followed by its dephosphorylation to isopentenol (Fig. 4). By producing an MVA pathway in *E. coli* and harnessing the promiscuous activities of two enzymes (phosphomevalonate decarboxylase and an endogenous phosphatase), it was possible to generate and test two bypasses for isopentenol biosynthesis (Kang et al., 2016). These branched from MVA-P and MVA (both MVA pathway intermediates), generating isopentenol-P and isopentenol *via* decarboxylation. Apart from eliminating the toxic effects of IPP accumulation such as growth inhibition, these bypasses avoid the kinetic limitation of mevalonate kinase, an important control point of the pathway.

Interestingly, all the examples we mentioned in this section illustrated that promiscuous enzyme activities are of vital importance for supporting the design of bypasses for this optimization purpose. In particular, retrosynthetic approaches can aid in the identification of those candidates to test for the activity of interest.

2.5. Uncoupling precursors from biomass synthesis for production

Another emerging use of bypasses for rewiring the host metabolism focuses on re-designing synthesis of specific biosynthetic precursors. Many examples are known in literature where metabolism has been rewired into new architectures that better support precursor supply for bioproduction, both at level of central and secondary metabolism (Lee et al., 2019; Park et al., 2018; Yu et al., 2019a; Zhu et al., 2021). However, many bioproduction routes depend on precursors that overlap with endogenous anabolic pathways. Frequently, this leads to direct competition for these precursors between cellular anabolism and bioproduction pathways. Nevertheless, in some cases such a precursor molecule is not essential for biomass buildup, but rather a metabolic intermediate *en route* towards an essential anabolic metabolite. In principle, the product precursor could be relieved from its anabolic role by introducing a synthetic bypass. Therefore, the metabolite would become fully available for bioproduction, with consequent increase in bioproduction while still being able to maintain biomass synthesis. Stretching this concept, we report here some examples where, guided by the need of streamlining synthesis of bioproduction precursors, new bypasses have redesigned anabolic reactions.

A recent example involves the heterologous expression of the Weimberg pathway in an *E. coli* Δ*icd* strain (deleted in the gene encoding isocitrate dehydrogenase) (Lu et al., 2021). This deletion was created to increase the intracellular pool of cis-aconitate available as precursor for itaconate biosynthesis. However, Δ*icd* also prevents synthesis of 2OG, which is essential to make glutamate and other amino acids. To allow for 2OG synthesis independent of cis-aconitate, the Weimberg pathway connecting xylose to 2OG was established (Fig. 8a). Through this route, the engineered strain could also generate the NADPH necessary to drive the conversion of 2OG into glutamate. The resulting strain was able to grow in a mixture of xylose and glycerol, and could produce itaconate in the g/L range (Lu et al., 2021).

Another example to release a native precursor from its anabolic function to exclusively support bioproduction involves the ethylmalonyl-CoA pathway (EMCP). The EMCP is present in several Proteo- and Actinobacteria and is used as an anaplerotic route to synthesize essential C4 metabolites when growing on C1 and C2 substrates. This metabolic pathway is also biotechnologically relevant, because it includes many thioesters of mono- and di-carboxylic acids as

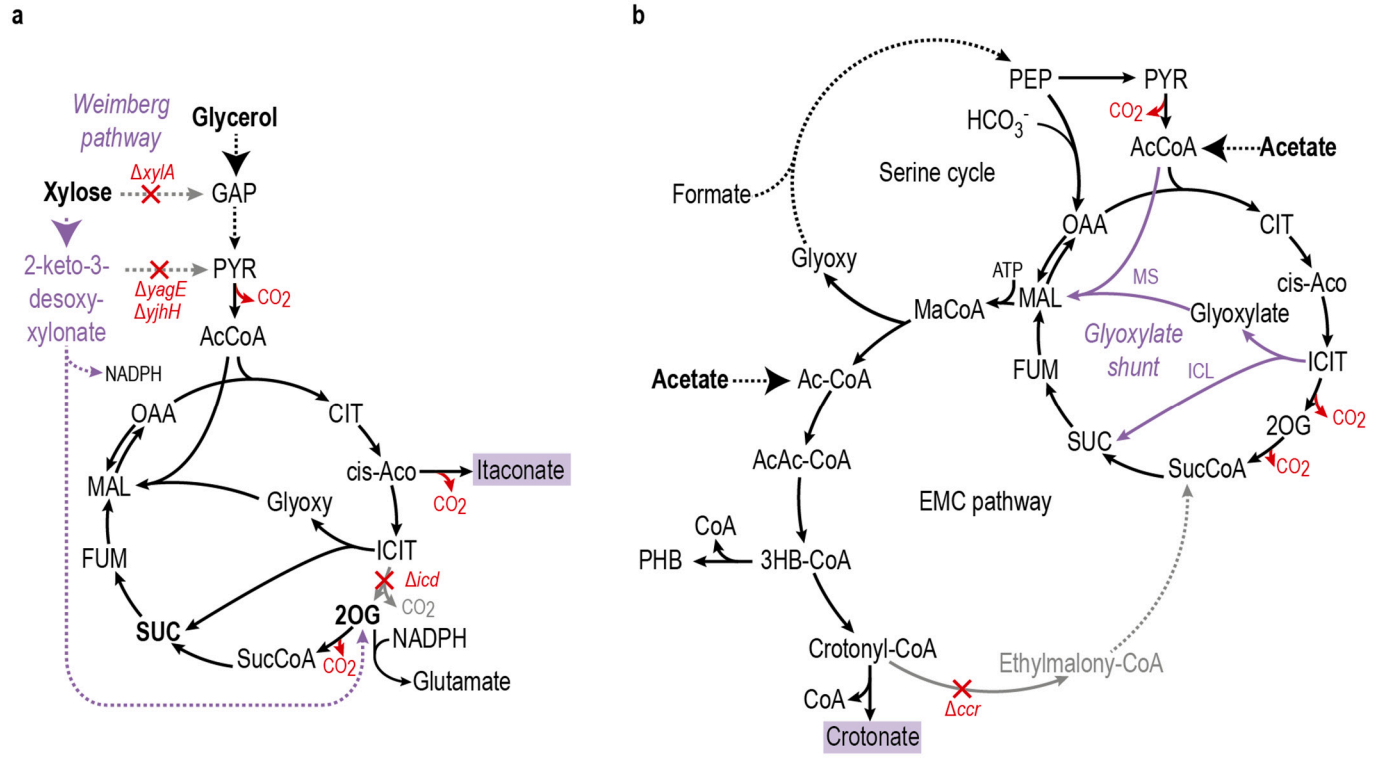

Fig. 8. Bypasses for redesigning precursors supply. a) Expression of a Weimberg pathway releases glutamate auxotrophy (Δ*icd*), which has been engineered for increasing itaconate synthesis (purple box) in *E. coli*. The Weimberg pathway (purple dashed arrows) also provides NADPH, which is oxidized in the conversion of 2OG into glutamate. Deletion of *xylA*, *yagE*, and *yjhH* prevents xylose to feed lower glycolysis. b) Transformation of the ethylmalonyl-CoA pathway (EMCP) into a bioproduction route in *Methylobacterium extorquens* (Schada Von Borzyskowski et al., 2018). When growing on acetate, the EMCP functions as an anaplerotic route, by feeding intermediates in the TCA cycle for biomass formation. By engineering a heterologous glyoxylate shunt (purple arrows), it was possible to release the EMC pathway from its anaplerotic role and, by deleting *ccr*, this route could be harnessed for the synthesis of biotechnological relevant compounds, such as crotonate (purple box). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intermediates, which can be interesting as platform chemicals. In an effort to tap these interesting EMCP intermediates for bioproduction, the central metabolism of *Methylobacterium extorquens* was redesigned by establishing a heterologous glyoxylate shunt (Schada Von Borzyskowski et al., 2018). This anaplerotic shunt, which is present in many organisms, but absent in *M. extorquens,* became the new anaplerotic route from acetate in an EMCP knockout strain (Fig. 8b). Through this approach, authors could release the EMCP from its anaplerotic function, while generating stoichiometric amounts carboxylic acid precursors through the glyoxylate route. As an example (Fig. 8b), the EMCP was harnessed for the production of the value-added compound crotonate (Schada Von Borzyskowski et al., 2018).

Many biotechnological processes present the challenge of competition for precursors between anabolic and production pathways. A widely used solution in metabolic engineering for obtaining high product titers is to uncouple growth and production phases using two-stage cultivations. In these setups, one can first allow the precursor to supply biomass synthesis, and later maximize the flux towards product formation when growth is inactivated. However, this requires tight, time-dependent control of enzyme production. As a valuable alternative, we propose the engineering of cell factories using bypasses that release a native precursor from its anabolic role, being fully available for bioproduction in, *e.g.*, continuous processes.

2.6. Altering redox cofactor types

Providing cofactors for redox reactions is vital for driving carbon flux towards the synthesis of desired compounds (Aslan et al., 2017; Davy et al., 2017). Many metabolic engineering approaches are described for enhancing cofactor availability, *e.g.*, by removal of competing pathways that might consume that specific cofactor. However, some bioproduction routes might require a different type of cofactor than the one that the classic metabolic architecture provides. Alternatively, completely new electron transfer functionalities can be designed and implemented for redox reactions, which are independent from the host's metabolic network. We argue that these engineering approaches can be motivated by one or more of the design principles presented in the previous sections. Nevertheless, we proposed to describe this approach as a stand-alone optimization purpose due to the increasing attention that the field for redox engineering is receiving within the metabolic engineering community.

2.6.1. Replacing native redox cofactors

The first category of cofactor swapping we describe focuses on the replacement of endogenous redox carrier. Some archetypal examples of this sort consist in changing cofactor generation from NADH to NADPH in glucose catabolism. NADPH usually drives anabolism and increasing the rate of its recovery can enhance the production of several added-value products, *e.g.*, isoprenoids, L-lysine, or L-glutamate. Several enzymes mediate NADPH regeneration, some of which can be coupled to central carbon metabolism (Spaans et al., 2015). Some of these reactions constitute bypasses of the textbook-example of glycolysis, the EMP pathway. The first example consists in rerouting the carbon flux *via* the ED pathway by interrupting the EMP pathway by deleting phosphoglucoisomerase (*pgi*). This inactivation can be combined to the overproduction of the key ED pathway enzyme: glucose 6-phosphate 1-dehydrogenase (ZWF), which converts G6P to 6-phosphogluconolactone (Fig. 9a). One additional NADPH molecule can be harvested through oxidative decarboxylation of 6-phosphogluconate to Ru5P *via* GND, corresponding to the first step of the oxidative PPP (Fig. 9a).

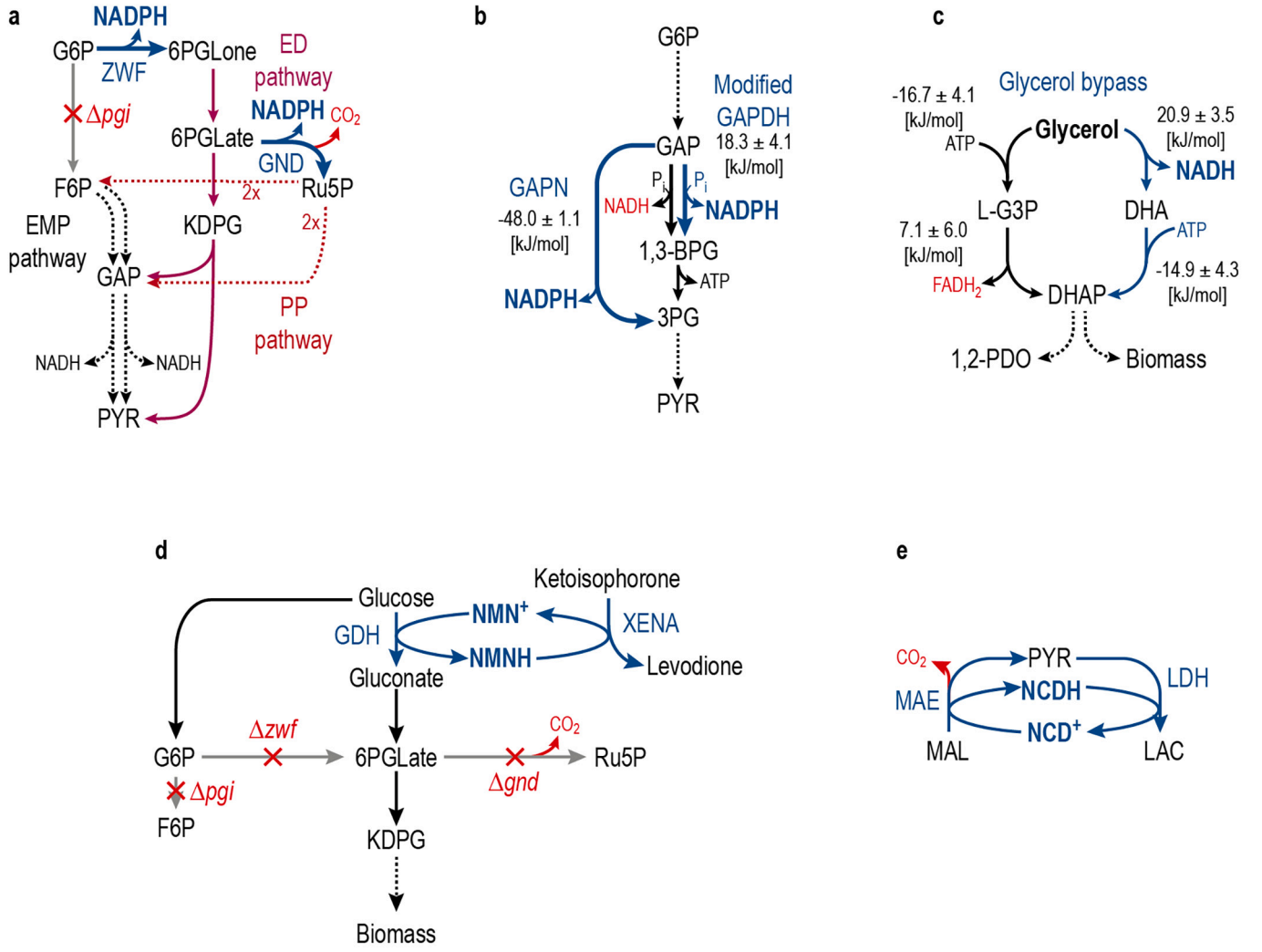

Fig. 9. Bypasses for altering the redox cofactor types (dark blue arrows). a) Deviating the carbon flux in glucose catabolism from the EMP pathway to the ED and PP pathways allows to harvest reducing power in the form of NADPH (dark blue arrows). Deletion of *pgi* in combination with ZWF and GND overproduction is often used for this purpose. It is worth highlighting that the oxidative decarboxylation of 6-phopshogluconate (6PGLate) to ribulose 5-phosphate (Ru5P) determines results in a stoichiometric carbon loss of one CO_2. This strategy is a common practice in many prokaryotic and eukaryotic cell factories. b) Engineering of NADPH generating reactions around the glyceraldehyde 3-phosphate (GAP) node. The engineering of GAP dehydrogenase (GAPDH) for improved specificity towards NADPH presents a $\Delta_rG'^m > 0$ [kJ/mol] and is thus unfavorable under physiologic conditions unless a strong NADPH sink is provided. Instead, the use of GAPN presents a $\Delta_rG'^m < 0$ [kJ/mol], although this increased free energy of the reaction comes at the sacrifice of harvesting ATP in the conversion of 1,3-bisphosphoglycerate (1,3-BPG) into 3-phosphoglycerate (3PG). The first examples of NADPH generation at the GAP node were described in *E. coli*. The numerical values refer to the $\Delta_rG'^m$ of the different reactions. c) Engineering of an alternative glycerol assimilation pathway in *S. cerevisiae* for the generation of NADH instead of $FADH_2$. This novel assimilation route proved to support growth and production of 1,2-propanediol (1,2-PDO) in yeast (Islam et al., 2017; Klein et al., 2016). The numerical values refer to the $\Delta_rG'^m$ of the different reactions. d, e) Use of non-canonical cofactors allows to funnel electron transfer from substrate to product. d) Use of nicotinamide mononucleotide (NMN^+) as electron carrier in combination with the coupling of the oxidizing reaction of glucose dehydrogenase (GDH) to the reductive reaction catalyzed by enoate reductase (XENA). This approach allowed to couple *E. coli* growth to the reduction of ketoisophorone into the pharmaceutical intermediate levodione (Black et al., 2020). e) Engineering of malic enzyme (MAE) and lactate dehydrogenase (LDH) for increased specificity towards nicotinamide cytosine dinucleotide (NCD^+) allowed to generate an electron circuit for the conversion of malate (MAL) into lactate (LAC) and nicotinamide cytosine dinucleotide (NCD^+) (Wang et al., 2021). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the lower EMP pathway, oxidation of GAP towards pyruvate occurs through the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which usually generates NADH and glycerate 1,3-bisphosphate (1,3-BPG). Next, the latter product can be used to generate ATP *via* phosphoglycerate kinase (PGK). For synthesizing some products (*e.g.* glutamate, L-lysine, isoprenoids), it is relevant to generate NADPH instead of NADH *via* GAPDH. This justified the replacement of the native NADH-dependent *E. coli* GAPDH with a heterologous NADPH-dependent one from *Clostridium acetobutylicum* (Martínez et al., 2008). Similarly, the endogenous GAPDH of *C. glutamicum* was rationally engineered for changing its cofactor specificity to $NADP^+$ (Bommareddy et al., 2014). Both cases reported a significant increase in the production of molecules relying on NADPH consumption for their synthesis (ε-caprolactone, lycopene, and L-lysine). However, the thermodynamic drive of this reaction is not favorable under physiologic conditions ($\Delta_rG'^m$ of 18.3 ± 7.0 kJ/mol) (Fig. 9b). Therefore, for assuring activity of this enzyme and maintaining cellular homeostasis, a still active NAD-variant is likely to be required which will continue in converting GAP while generating NADH. Alternatively, a strong NADPH sink should be provided, as with the synthesis of added-value molecules (*e.g.*, glutamate, L-lysine, isoprenoids) through NADPH-consuming pathways. This latter approach would directly reduce the $\Delta_rG'^m$ of the NADPH-dependent GAPDH

reaction in central metabolism by decreasing the concentration of one of the reaction products *i.e.*, NADPH. However, there is limit to this approach as the decrease of the intracellular NADPH/$NADP^+$ ratio can only be realized within certain boundaries to not disturb other cellular processes relying on this cofactor. Alternatively, by sacrificing one step of substrate-level phosphorylation, the non-phosphorylating, NADPH-dependent GAP dehydrogenase (GAPN) can be used for the same purpose. This enzyme catalyzes the direct conversion of GAP into 3PG, thereby bypassing 1,3-BPG formation and the consequent ATP generation step (Fig. 9b). This results in a higher thermodynamic drive of the reaction corresponding to highly negative $\Delta_rG'^m$, which can therefore support spontaneous NADPH generation in lower glycolysis. Use of GAPN can be coupled to growth (Lindner et al., 2018), and it has been widely used for enhancing biosynthesis of NADPH-derived products, such as poly-hydroxybutyrate (PHB) and L-lysine (Centeno-Leija et al., 2014; Takeno et al., 2010). In an attempt of reducing glycerol synthesis as byproduct of ethanol fermentation, expression of GAPN in *S. cerevisiae* allowed to decrease the NADH generated within glucose catabolism (generating NADPH instead), eventually reducing the amount of glycerol produced by almost 50% (Guo et al., 2011).

Another example of change in cofactor specificity involves glycerol catabolism (Fig. 9c). This molecule is the main by-product of biodiesel production, accounting for approximatively 10% (*w*/w). Use of glycerol as bio-based feedstock is therefore a valuable opportunity for the circular economy (Dobson et al., 2012). Moreover, its higher degree of reduction per carbon ($\gamma = 4.67$) compared to glucose ($\gamma = 4$) justifies its use for the synthesis of reduced molecules, *e.g.* 1,2- or 1,3-propanediol (1,2- or 1,3-PDO, $\gamma = 5.33$) (Dobson et al., 2012; Islam et al., 2017). However, to achieve the maximum theoretical yield, it is important to maximize the electron transfer from substrate to product. Efficient electron transfer is possible when using a redox carrier with low reduction potential ($E'^0 << 0$). In *S. cerevisiae*, endogenous glycerol assimilation occurs *via* a two-step pathway, which firstly activates glycerol to L-glycerol 3-phosphate, and then oxidizes it in the mitochondria to dihydroxyacetone phosphate (DHAP) *via* a FAD^+-dependent reaction (Klein et al., 2016). However, reduced $FADH_2$ is not an ideal redox carrier for driving the synthesis of reduced fermentation products, because of its relatively high reduction potential (E'^0 $FAD^+/FADH_2$ = -220 mV). Instead, a cofactor such as NADH would harvest a bigger amount of substrate's reducing power thanks to its more negative reduction potential (E'^0 NAD^+/NADH = -320 mV), maintaining the electrons available for further intracellular redox reactions towards product formation. As DHAP is a glycolysis intermediate, it can be isomerized to GAP, which is also a biomass precursor. In order to harvest reducing power from glycerol, its oxidation to DHAP was redirected in *S. cerevisiae* through a synthetic pathway consisting of a heterologous NAD^+-dependent glycerol dehydrogenase, and an endogenous dihydroxyacetone kinase (Klein et al., 2016) (Fig. 9c). Implementation of this metabolic bypass while inactivating the endogenous route allowed to increase the yield of NADH (Klein et al., 2016). Direct evidence of the bypass efficacy led to high growth rate on glycerol for the engineered strain (Klein et al., 2016). The biotechnological exploitation of this bypass was followed by the proof-of-concept of 1,2-PDO production in yeast *via* this synthetic assimilation route (Islam et al., 2017).

When discussing the use of a bypass for the replacement of cofactor types, it is relevant to consider the associated effect on the reaction energetics. As explained above, the reduction potential E'^0 of a cofactor expresses its tendency to acquire electrons. When moving *e.g.*, from FAD^+ (E'^0 = -220 mV) to NAD^+ (E'^0 = -320 mV) as redox equivalent, one would expect less energy to be dissipated while reducing NAD^+ than FAD^+. Consequently, this would result in a lower thermodynamic drive (ΔG') for the reaction generating NADH compared to the one producing $FADH_2$. Indeed, the $\Delta_rG'^m$ of the two reactions within glycerol catabolism are significantly different, with $FADH_2$ production being more favorable: 20.9 ± 3.5 kJ/mol and 7.1 ± 6.0 kJ/mol for NADH and $FADH_2$ generation, respectively (Fig. 9c). Another consideration involves the ratio between the oxidized and the reduced form of the redox equivalent as *e.g.*, in the case of cofactor replacement in GAPDH. In *E. coli*, the endogenous reaction relies on NAD^+ as electron acceptor, where the intracellular NAD^+/NADH ratio is 19 (Andersen and von Meyenburg, 1977). Therefore, there is a high abundance of NAD^+ (substrate) which can drive the reaction. Instead, when modifying GAPDH to accept $NADP^+$, the enzyme must cope with a less favorable $NADP^+$/NADPH ratio of 1.2 (Andersen and von Meyenburg, 1977). This results in a lower thermodynamic drive which, as explained above, renders the reaction more challenging. In summary, we pose that is crucial to consider the thermodynamic implications when designing a bypass for changing the redox cofactor type.

2.6.2. Introducing non-canonical redox cofactors

Another emerging approach for maximizing the electron transfer from substrate to product involves the design of new electron transfer functionalities which are completely independent from the host's endogenous redox network (Weusthuis et al., 2020). This can be enabled using 'non-canonical cofactors' (Black et al., 2020): synthetic electron carrier molecules which generate an orthogonal electron circuit, and therefore operate in parallel to the host's network. In principle, non-canonical cofactors prevent electron dissipation, maximize transfer of reducing power from substrate to product and, therefore, can aid in approaching maximum theoretical yields for product formation (Weusthuis et al., 2020).

The use of non-canonical cofactors requires the synthesis of novel redox carrier, as well as the adaptation of native enzymes for increased specificity (k_{cat}/K_M) towards the cofactors. One breakthrough in this research field was recently obtained when developing a new redox system based on nicotinamide mononucleotide (NMN^+) (Black et al., 2020). This cofactor, which can now be synthesized *in vivo* after *E. coli* engineering, is structurally similar to $NADP^+$, but lacks an AMP moiety. A glucose dehydrogenase enzyme (GDH) was engineered in its amino acid composition to enhance its specificity for NMN^+ while reducing it for $NADP^+$. The activity of GDH was then coupled to a NMNH-oxidizing enzyme enoate reductase (XENA) in a Δ*pgi* Δ*zwf* Δ*gnd E. coli* strain, which could release free NMN^+, and therefore support the conversion of glucose to gluconate for growth (Fig. 9d) (Black et al., 2020). To provide enough NMN^+ for the desired activity, its buildup had to be engineered *via* heterologous gene expression. Eventually, by using this approach, authors could demonstrate the reduction of ketoisophorone into the pharmaceutical intermediate levodione. Recently, a variant of GAPN with improved specificity towards NMN^+ was engineered and used to support NMN^+-dependent activity within the EMP pathway (King et al., 2022).

Another non-canonical cofactor that has been engineered and proven to work *in vivo* is nicotinamide cytosine dinucleotide (NCD^+), an analog of NAD^+ where a cytosine replaces the adenine in the cofactor backbone. This synthetic non-canonical cofactor (supplemented in the medium and transported intracellularly) proved to catalyze the transfer of electrons in a circuit involving modified phosphite dehydrogenase and malate dehydrogenase as redox enzymes. The latter could selectively catalyze the reductive carboxylation of pyruvate into malate using the electrons from phosphite (Wang et al., 2017a). In a more recent report from the same group, a lactate dehydrogenase from *Lactobacillus helveticus* was engineered for an increased dependence towards NCD^+ over NAD^+, and then expressed in *E. coli* (Liu et al., 2020). The improved specificity was demonstrated by analysis of k_{cat} and K_M and, *in vivo*, by measuring reduced titers of lactate when NADH was the only cofactor available. Finally, the biosynthetic pathway for NCD^+ was engineered *in vivo*, and supported the electron transfer within a metabolic circuit converting malate (functioning as both carbon source and electron donor) into lactate (Fig. 9e) (Wang et al., 2021).

Other types of non-canonical cofactors apart from nicotinamide types have been proposed in literature (Paul et al., 2021; Wang et al., 2017b; Weusthuis et al., 2020). We expect them to be implemented *in*

vivo in the coming years to realize complete orthogonal electron transfers within the metabolic network.

3. Discussion

At its origin, the field of metabolic engineering considered metabolism as a robust network (Stephanopoulos and Vallino, 1991), and approaches like control analysis have been employed as an investigation tool to optimize the carbon flux towards a product of interest (Fell, 1998). Our current ability to reconnect metabolic nodes in novel ways demonstrates that we are witnessing a new era in metabolic engineering, where we can operate at a higher freedom of design and exert a higher degree of control over metabolic plasticity. In line with this view, we formulated six design principles motivating the novel connection of metabolic nodes *via* bypasses. As the bypasses described in literature are constantly increasing, we mainly focused on the most striking examples established *in vivo*.

We should not forget that the central carbon metabolism has evolved into a 'minimal walk', where allocation of resources (*i.e.* protein and energetic investment) is optimized (Bar-Even et al., 2012; Noor et al., 2010). Therefore, deviation from this minimal walk may be associated to a metabolic cost (Table 1), and predicting the associated drawbacks becomes vital when implementing bypasses in cell factories. For example, we observed that bypasses increasing stoichiometric gain during substrate catabolism or assimilation are inevitably limited in the reducing energy and ATP they generate. This is an inherent trait of, *e.g.*, the NOG (Bogorad et al., 2013; Lin et al., 2018) and the Weimberg pathway (Radek et al., 2014). Also, when realizing carbon-positive bypasses using CO_2, a net investment of reducing equivalent is required. Similarly, as briefly reasoned in Section 2.6.1, producing a cofactor type with a higher reduction potential might result in a lower Gibb's free energy change for that reaction. Another consistent metabolic cost was associated to overcoming thermodynamic barriers of reactions. In all the cases reported, ATP is consumed, as demonstrated while reverting the activity of malate synthase, citrate synthase (Mainguet et al., 2013), or for the establishment of a malonyl-CoA bypass (Lan and Liao, 2012).

Therefore, a clever metabolic design for a successful bypass should maximize the gain with its implementation, while limiting its drawbacks. In this regard, a possible strategy could consist of designing bypasses that, *e.g.*, allow to conserve energy during anaerobic fermentations -a form of stoichiometric gain in terms of ATP- (Folch et al., 2021; Yu et al., 2019b). Also, the implementation of bypasses could be coupled to dynamic regulation of metabolic fluxes (Hartline et al., 2021; Ni et al., 2021), while dividing the cultivation between growth- and production-phases (Raj et al., 2020). In this way, the designer can switch between a growth-phase (where the objective is to accumulate biomass) to a production-phase (where the bypass can be 'activated' or the competing pathway 'inactivated'). Nevertheless, as discussed in Section 2.5, the use of bypasses that release a product precursor from its anabolic role can allow to explore new production processes in continuous mode.

The energetic cost for the protein synthesis of a heterologous bypass is expected to be of the same order of magnitude as overexpressing the gene encoding for a rate-limiting enzyme: about 30 ATP molecules per amino acid (Wagner, 2005). These values will be particularly similar when using the same strength promoters or ribosome binding sites to control gene expression. Therefore, the metabolic burden of enzyme production is not going to be a critical variable when implementing a bypass. Instead, the design should focus mainly on the design principle (s) to follow to obtain flux increase through a bottleneck.

Recently, the design of new bypasses for the conversion of C1 substrates formate, formaldehyde, and methanol into C2 products demonstrated that it is possible to completely circumvent the bow-tie structure of metabolism for bioproduction (Chou et al., 2021). This design has been described as orthogonal by the authors and, in principle, can support production also in a growth-independent manner. Alternatively, co-utilization of the bypass together with the endogenous, antagonistic pathway can also result in a synergistic effect, as demonstrated for both substrate catabolism (Lee et al., 2021; Meadows et al., 2016; Wang et al., 2018) and product biosynthesis levels (Orsi et al., 2020a; Yang et al., 2016).

Many of the bypasses included in this review were realized *in vivo* either by rational design or emerged as consequence of enzyme evolution (Noda-Garcia et al., 2018). In the latter case, the use of growth-coupled selection schemes (Orsi et al., 2021; Trinh and Mendoza, 2016; Wenk et al., 2018) proved to be advantageous, since it acted as selective pressure for the screening -or evolution- of the enzymatic activity of interest (Chen et al., 2018; Claassens et al., 2019b; Kirby et al., 2015; Mainguet et al., 2013; Ran et al., 2004). Nevertheless, not always these approaches were successful, as illustrated *e.g.* in the case of an attempted synthetic glycerol assimilation pathway (Lindner et al., 2020). Apart for canonical reactions, the same *in vivo* approach could be harnessed to select for bypasses relying on promiscuous enzymatic activities capable of rewiring central carbon metabolism (He et al., 2020; Krüsemann et al., 2018).

Another emerging, complementary approach to the direct implementation of *in vivo* bypasses consists of the *in vitro* study of enzyme activities through cell-free systems (Dudley et al., 2015). In particular, use of cell-free approaches allows to carefully control the reaction environment, while generally avoiding constraints such as *i.e.* limited metabolite concentrations, and osmotic pressures. This in general results in higher yields and productivities than in microbial cells (Claassens et al., 2019a). Therefore, the *in vitro* condition represents a simpler environment where to assess enzyme mechanisms and kinetics towards the design of new bypasses. This is particularly true for new-to-nature pathways that can be designed *via* 'mix-and-match' approaches (Erb et al., 2017). Some remarkable examples of this sort reported in literature are the methanol condensation cycle (Bogorad et al., 2014), and the tartronyl-CoA pathway (Scheffen et al., 2021). The former combines part of the RuMP and NOG pathways for efficient n-butanol production, whereas the latter is an example of carbon-positive bypass aiming at tackling photorespiration (Trudeau et al., 2018). Possibly, the *in vivo / in vitro* approaches can be further coupled for improving titers and volumetric productivities (Rasor et al., 2021).

A holistic approach for engineering cell factories (Aslan et al., 2017) is recommended also when designing metabolic bypasses. This can benefit from the integration of systems biology as an aiding discipline for optimizing and de-regulating bioproduction (Pandit et al., 2017; Yu et al., 2019a). In fact, many of the reported examples demonstrated that use of bypasses in the upstream part of the metabolism can have repercussions in the production titers. Altogether, the emerging concept of metabolic bypassing constitutes an additional tool in the box for the design of superior cell factories. Ultimately, their use for the design of complete orthogonal pathways (Chou et al., 2021; Mampel et al., 2013; Pandit et al., 2017) in combination with the employment of non-native cofactors (Weusthuis et al., 2020) could represent an important step for advancing industrial biotechnology by means of synthetic biology.

Author contributions

All authors were involved in the initial conceptualization of the manuscript. E.O. wrote the manuscript that was critically reviewed by all authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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