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Metabolic engineering strategies to enable microbial utilization of C1 feedstocks

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One-carbon (C1) substrates are preferred feedstocks for the biomanufacturing industry and have recently gained attention owing to their natural abundance, low production cost and availability as industrial by-products. However, native pathways to utilize these substrates are absent in most biotechnologically relevant microorganisms. Recent advances in synthetic biology, genome engineering and laboratory evolution are enabling the first steps towards the creation of synthetic C1-utilizing microorganisms. Here, we briefly review the native metabolism of methane, methanol, CO_2, CO and formate, and how these C1-utilizing pathways can be engineered into heterologous hosts. In addition, this review analyses the potential, the challenges and the perspectives of C1-based biomanufacturing.

As the global shift to greater accountability in sustainable production takes hold, led by the change in energy generation away from fossil sources, manufacturing is poised to move towards greater sustainability, which includes less reliance on the energy-intensive and greenhouse gas (GHG)-emissions-heavy base of the current operation[1]. Biomanufacturing, in which microbial cell factories provide a conversion platform for low-cost feedstocks into higher-value products such as alcohols, lipids, amino acids and organic acids, is stepping up to meet demand. Currently, industrial biomanufacturing relies heavily on sugar-containing feedstocks such as glucose and sucrose[2].

As biomanufacturing expands and accountability for sustainable practices increases, new feedstocks will be required to meet growth[3]. Although lignocellulose is an important source of non-food sugar feedstock, its recalcitrance and pre-treatment requirements limit its use in biomanufacturing. One-carbon (C1) compounds, chiefly carbon dioxide (CO_2), carbon monoxide (CO), methane (CH_4), methanol (CH_3OH) and formate (HCOOH), are substrates that could allow us to reshape industrial biomanufacturing away from sugar-based feedstocks (Fig. 1)[4–6]. Arguments for shifting the feedstock base to C1 compounds include the abundance and low cost of C1 compounds, as some are currently waste products, and preventing their release to the atmosphere may confer the additional benefit of slowing their impact on global warming, as some are potent GHGs. Furthermore, compared with glucose, some C1 compounds harbor a larger number of available electrons per C atom, and could deliver greater yields[7]. A key difference between C1 and sugar feedstocks is the lack of carbon–carbon bonds in C1 compounds, which means that all multi-carbon metabolic intermediates used for the synthesis of cellular constituents and energy in synthetic C1-utilizer organisms must be generated de novo via assimilatory metabolic pathways[8].

In recent years, researchers in synthetic biology and metabolic engineering have faced the challenge of shifting the core metabolism of industrial microbes away from their reliance on sugars to effective use C1 compounds as substrate, by building new pathways into established industrial biotechnology microbes. However, the development of natural C1-utilizing organisms for biomanufacturing is hampered by their low efficiency and the lack of synthetic biology tools and industrial familiarity with their use[9,10]. Among recent achievements, in 2019 the first eukaryotic (*Pichia pastoris*)[11] and prokaryotic (*Escherichia coli*)[12] cells able to synthetically fix CO_2 were developed (Fig. 2a). In 2020, the first *E. coli* cells able to grow on methanol[13] and formate[14] were achieved (Fig. 2b,c). Here, we summarize progress in the field—the biotechnological use of C1 substrates by microbial cells, engineering approaches to create synthetic C1-utilizing strains, and the challenges of and prospects for C1-based metabolic engineering—to provide a reference point for further exploiting the biotechnological potential of C1 substrates.

Methanol as a C1 substrate

In 2018, worldwide methanol production capacity reached 110 million metric tons[15]. Methanol is commonly produced via steam reforming of natural gas, from biomass-derived synthesis gas, or via hydrogenation of CO_2[16,17]. As there are abundant sources and flexible production processes, methanol is generally available at a lower price than sugar[18]. In addition, methanol is more reduced than glucose and theoretically could improve product yields[7].

Methanol is naturally utilized by methylotrophic prokaryotes and selected eukaryotes from *Pichia*, *Candida* and *Torulopsis* genera as their sole carbon source[10,19,20]. The first step of methanol metabolism in methylotrophs is oxidation to formaldehyde, catalyzed by alcohol oxidase (Aox) in yeasts or methanol dehydrogenase (Mdh) in prokaryotes (Fig. 3)[5,21–23]. Methanol oxidation in methylotrophic yeasts requires O_2 as an electron acceptor[8], whereas Mdh uses either pyrroloquinoline quinone (PQQ) or nicotinamide adenine dinucleotide (NAD^+) as dependent electron acceptors for gram-negative and thermophilic gram-positive methylotrophs, respectively[5]. As the product of methanol oxidation, formaldehyde, is toxic due to its propensity to react with and subsequently crosslink macromolecules such as proteins and DNA, it is quickly assimilated or dissimilated in methylotrophs[13,19,24].

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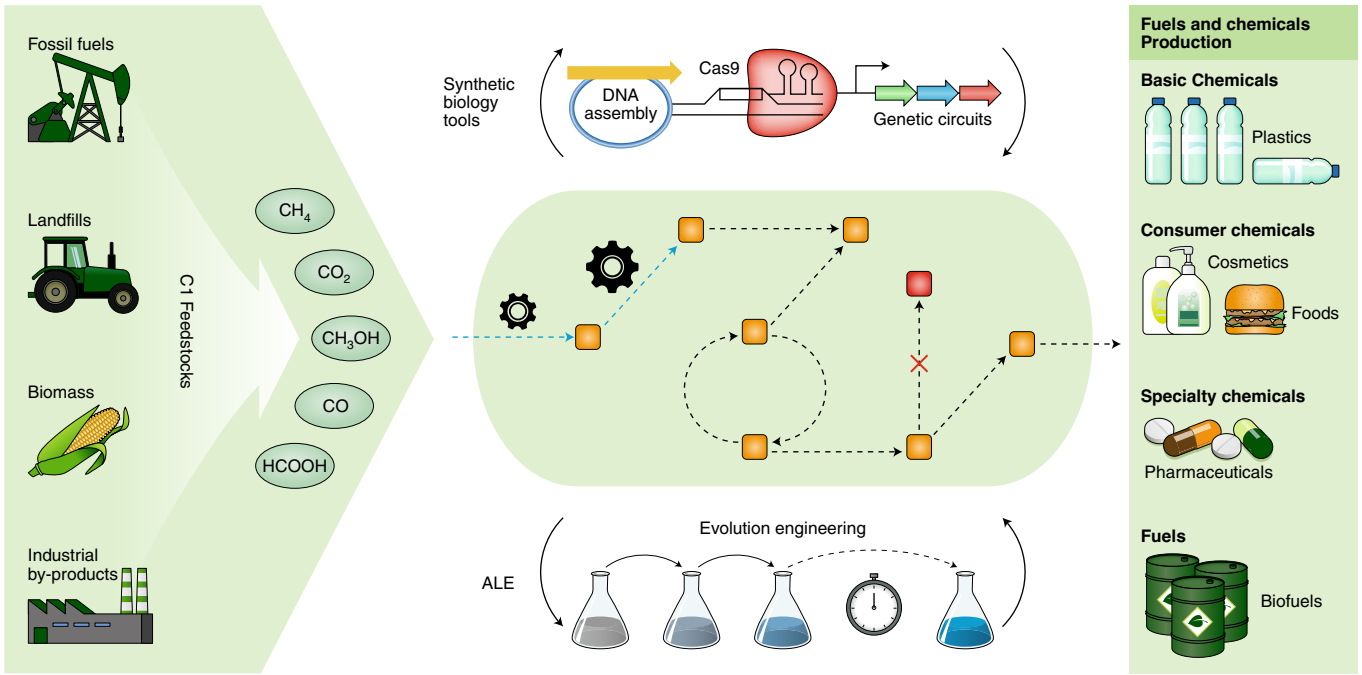

Fig. 1 | C1-based biomanufacturing. C1 compounds are low-cost abundant feedstocks that can be produced from diverse raw resources such as fossil fuel, industrial by-products and biomass. However, biomanufacturing is heavily dependent on sugar-based feedstock. In the past few years, C1 compounds have attracted attention as an alternative sustainable non-food feedstock for biomanufacturing, avoiding competition for food-based feedstocks. With the development of synthetic biology, genomic engineering and laboratory evolution, C1-based cell factories could be further developed to produce high-value products, thereby creating more industrial and commercial value. Middle panel shows metabolites from the substrate (C1) to the products (orange squares), competing metabolites (red squares), the C1 utilization pathway (blue dashed arrows), and other metabolic reactions (black dashed arrows).

The first step of formaldehyde assimilation in methylotrophic yeasts is its conversion to dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate (G3P) using xylulose 5-phosphate (Xu5P) as co-substrate (Fig. 3a). The products DHA and G3P are converted into Xu5P, completing the xylose–monophosphate cycle. All methanol assimilation steps occur within the peroxisomes and create G3P, one-third of which goes to the cytosol to create cell biomass and energy[25]. By contrast, Mdh oxidation of methanol in methylotrophic prokaryotes is conducted either in the periplasm for PQQ-dependent pathways or the cytosol for NAD^+-dependent pathways[26,27]. Once formed, there are two main assimilation routes for formaldehyde (Fig. 3b): the ribulose monophosphate pathway (RuMP) or the serine pathway. Via RuMP, formaldehyde is condensed with ribulose 5-phosphate (Ru5P) through two consecutive reactions catalyzed by 3-hexulose-6-phosphate synthase (Hps) and 6-phosphate-3-hexuloisomerase (Phi) to generate fructose 6-phosphate (F6P), an intermediate in common with yeast pathways[28]. F6P can enter into the Embden–Meyerhof–Parnas pathway (EMP), the Entner–Doudoroff (ED) pathway or the pentose phosphate pathway (PPP) to regenerate Ru5P[5,7]. Formaldehyde assimilation via the serine pathway is initiated through methylene tetrahydrofolate (methylene-H_4F) formation[29], which condenses with glycine to form serine, and is then converted to oxaloacetate, which links the serine cycle with the tricarboxylic acid (TCA) cycle through interconversion with malate[30]. In addition, methanol can also be assimilated by anaerobic microorganisms, such as some acetogens, which use the high yielded reductive acetyl-CoA pathway[31].

Dissimilation pathways are broadly present in microorganisms to consume endogenously generated formaldehyde[24]. In most of these cases, formaldehyde condenses spontaneously with a C1 carrier such as tetrahydrofolate (H_4F), tetrahydromethanopterin (H_4MPT), glutathione (GSH), bacillithiol or mycothiol (MSH) (Fig. 3a,b) to form a cofactor-bound C1 unit, which is converted to formate and eventually to CO_2, with energy generated in the form of NADH[32]. Notably, in *E. coli*, the formaldehyde dehydrogenase A (FrmA), involved in the dissimilation of formaldehyde to CO_2, is routinely deleted in synthetic methylotrophic *E. coli* to avoid the consumption and loss of carbon required for synthesis of cellular constituents and biomass[13,33–36].

Recent progress towards synthetic methylotrophy in *E. coli* has been considerable, with a foundation established through the identification of the most promising candidate genes for methanol metabolism from methylotrophs, namely *mdh, hps* and *phi*[37]. Isotope incorporation experiments with ^{13}C-methanol showed 40% assimilation of label into central carbon metabolites, especially hexose 6-phosphate (H6P) in *E. coli* expressing the three genes, demonstrating that the RuMP pathway established by Hps and Phi is functional[37]. Methanol oxidation and formaldehyde assimilation was improved by physically co-locating key enzymes including Mdh, Hps and Phi in a linked complex, resulting in a 50-fold increase in the conversion of methanol to F6P[38].

Once these methanol assimilation pathways were established, the research effort was directed to overcoming the challenges of using methanol as the sole carbon source for *E. coli* growth and energy. Cofactor imbalance is a key issue, as methanol oxidation via Mdh is inhibited as the cellular ratio of NADH to NAD^+ increases[39]. The conversion of methanol to formaldehyde was improved 3.6-fold by coupling this step to an NADH consumption cycle[38]. Alternatively, cellular NADH concentration was reduced by deletion of *maldh* encoding NAD^+-dependent malate dehydrogenase (Fig. 4a), which mimicked natural methylotrophs in lowering TCA cycle activity[40].

In addition to cofactor imbalance, NAD^+ oxidation of methanol is endergonic and requires low formaldehyde levels to maintain the forward direction of the reaction[39]. Xylose addition to the media of engineered *E. coli* markedly increased Ru5P concentration, and as an essential co-substrate with formaldehyde, Ru5P promoted

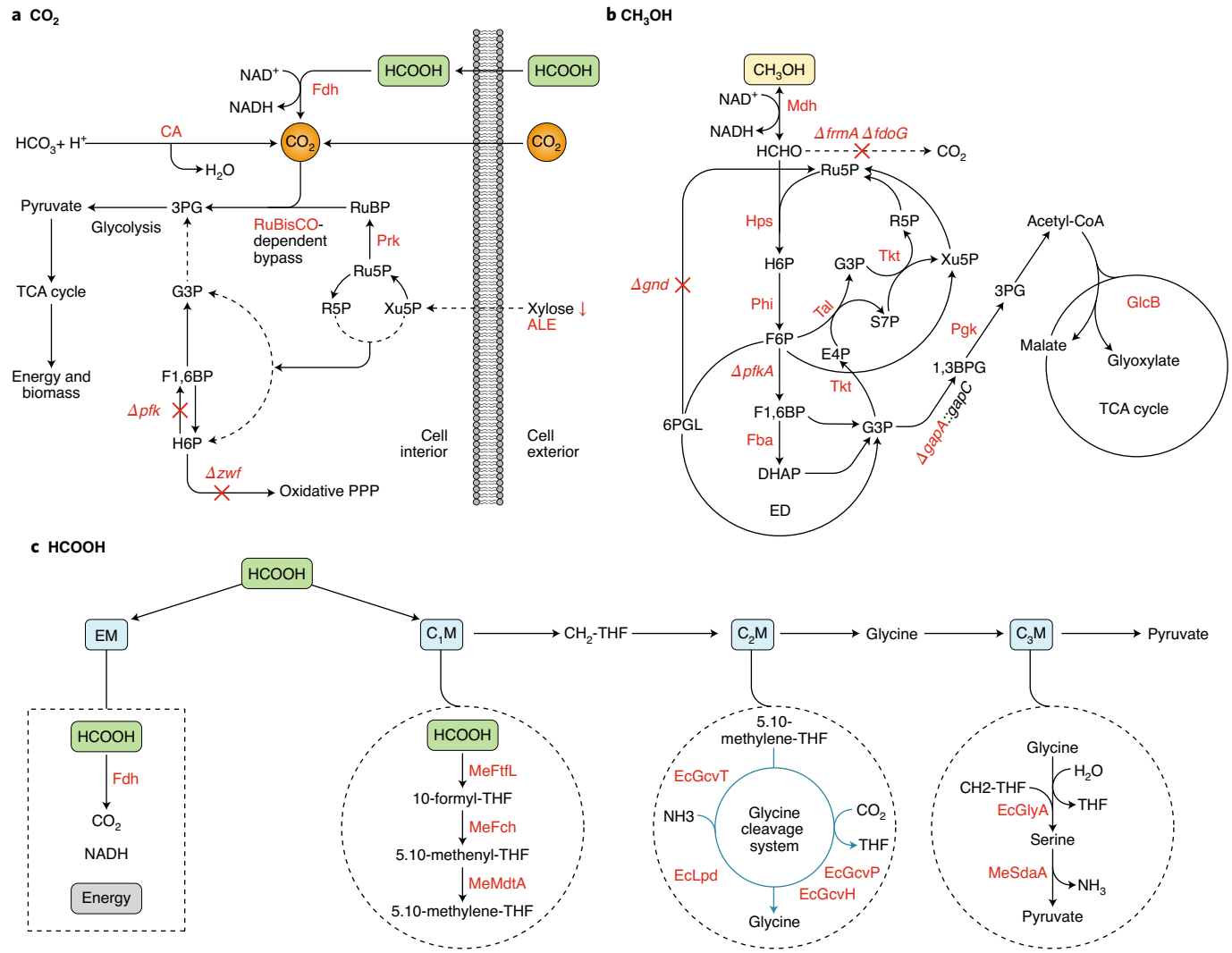

Fig. 2 | Examples of successful approaches to enable C1 substrate utilization in *E. coli*. **a**, All biomass carbon in engineered *E. coli* is formed from CO_2 by heterologous overexpression of RuBisCO, Prk, CA and Fdh to build a synthetic CBB cycle and utilize formate to generate sufficient reducing power. The deletion of *pfk* and *zwf* made cellular growth dependent on carboxylation by RuBisCO. Growth based solely on CO_2 uptake was achieved following an ALE process decreasing the amounts of externally added xylose[12]. **b**, Engineered *E. coli* grown on methanol feedstock through expression of heterologous RuMP pathway genes, deletion of *frmA* and *pfk*, and replacement of *gapA* with *gapC*, followed by ALE[13]. **c**, *E. coli* grown on formate as its carbon source after engineering four modules of the reductive glycine pathway and performing short-term evolution[14]. Metabolites: ribulose-5-phosphate (Ru5P), ribose 5-phosphate (R5P), ribulose-1,5-bisphosphate (RuBP), Xu5P, 3-phosphoglycerate (3PG), G3P, F1,6BP, H6P, F6P, DHAP, DHA, 6-phosphogluconate (6PG), THF, erythrose 4-phosphate (E4P), sedoheptulose 7-phosphate (S7P), 6-phosphogluconolactonase (6PGL), 1,3-bisphosphoglyceric acid (1,3BPG). Enzymes: Fdh, Pfk, Prk, Zwf, CA, RuBisCO, Mdh, Hps, Phi, FrmA, formate dehydrogenase (FdoG), transaldolase (Tal), transketolase (Tkt), glyceraldehyde-3-phosphate dehydrogenase A (GapA), glyceraldehyde-3-phosphate dehydrogenase C (GapC), fructose-bisphosphate aldolase (Fba), 6-phosphogluconate dehydrogenase (Gnd), phosphoglycerate kinase (Pgk), malate synthase G (GlcB), formate-THF ligase from *M. extorquens* AM1 (MeftfL), 5,10-methenyl-THF cyclohydrolase from *M. extorquens* AM1 (MeFchA), 5,10-methylene-THF dehydrogenase (MeMtdA), lipoamide dehydrogenase from E. *coli* (EcLpd), glycine dehydrogenase from *E. coli* (EcGcvP), aminomethyltransferase from *E. coli* (EcGcvT), lipoic acid-containing protein from *E. coli* (EcGcvH), serine hydroxymethyltransferase from *E. coli* (EcGlyA), serine deaminase from *M. extorquens* AM1 (MeSdaA). Blue arrows represent the glycine cleavage system; dashed straight lines are multiple enzymatic reactions; red labels represent heterologously expressed genes, deleted genes, and genes that after evolution were upregulated or found in high copy number.

formaldehyde assimilation. However, this action concurrently decreased methanol oxidation rate by increasing NADH formation. Instead, iodoacetate was used to block lower glycolysis in an engineered strain of *E. coli* in which the phosphorylation of fructose 1,6-bisphosphatase (F1,6BP) was reversed and carbon flux redirected to Ru5P formation (Fig. 4b), which resulted in a fourfold increase in Ru5P and threefold reduction in formaldehyde concentrations, demonstrating that an increased pool of Ru5P can lead to higher methanol assimilation[39]. An alternative strategy of heterologous expression of the PPP pathway from *Bacillus methanolicus* coupled with the knockout of phosphoglucose isomerase (Pgi), rerouted glucose catabolism through the PPP rather than via glycolysis and enhanced Ru5P generation in cells grown on methanol and glucose (Fig. 4c)[35]. Deletion of *rpe* encoding ribulose-phosphate 3-epimerase or *rpiAB* encoding R5P isomerases A and B allowed exogenous xylose or ribose to be used directly to increase Ru5P production, and following adaptive engineering, achieved growth on an approximately 1:1 molar ratio of methanol:xylose as co-substrates (Fig. 4d,e)[41].

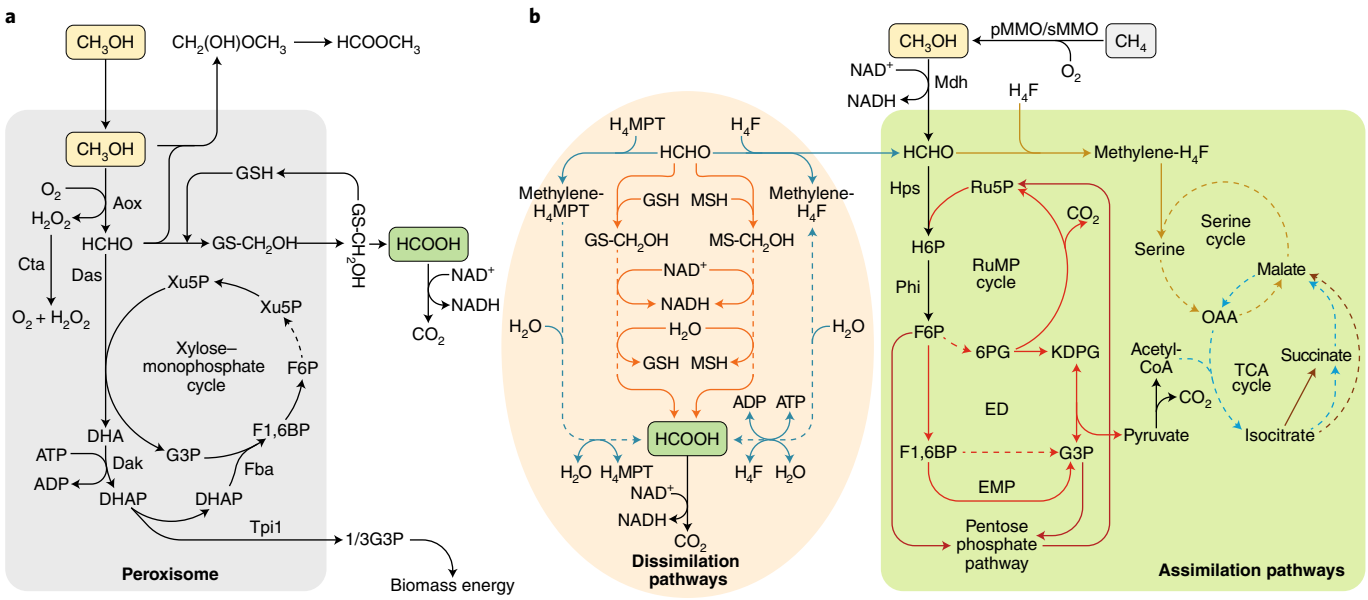

Fig. 3 | Methanol- and methane-utilization pathways in native organisms. a, Methanol assimilation and dissimilation pathways in native methylotrophic yeast. **b**, Methanol assimilation and dissimilation in native methylotrophic prokaryotes and aerobic oxidation pathway of methane in native methanotrophs. Pentose phosphate pathway (dark red), the RuMP cycle, the ethylmalonyl-CoA (EMC) pathway and ED pathway (red), serine cycle (yellow), TCA cycle (cyan), glyoxylate cycle (brown), H_4MPT- and H_4F-dependent formaldehyde assimilation pathway (blue), GSH- and MSH-dependent formaldehyde assimilation pathway (orange). Dashed arrows represent multiple enzymatic reactions. Metabolites: H6P, F6P, F1,6BP, ribulose 5-phosphate (Ru5P), Xu5P, G3P, DHAP, DHA, S-hydroxymethyl glutathione (GS-CH2OH), 2-keto-3-deoxy-6-phosphogluconate (KDPG), 6PG, tetrahydrofolate (H4F), tetrahydromethanopterin (H4MPT), GSH, bacillithiol or MSH, S-hydroxymethyl mycothiol (MS-CH2OH), methylene-tetrahydromethanopterin (methylene-H4MPT), methylene-tetrahydrofolate (methylene-H4F). Enzymes: Aox, Das, catalase (Cta), dihydroxyacetone kinase (Dak), Mdh, Hps, Phi, membrane-bound methane monooxygenase (pMMO), cytoplasmic methane monooxygenase (sMMO); OAA, oxaloacetate.

Alternative synthetic methylotroph strategies avoided the need for Ru5P co-substrate regeneration by designing novel pathways. For example, linear methanol assimilation was introduced in *E. coli* by expression of Mdh and formolase (Fls), which converted three formaldehyde molecules to DHA (via the carboligation reaction) that was then incorporated into cellular biomass[33]. Furthermore, a functional hybrid formaldehyde assimilation pathway was constructed by co-expression of Mdh from *Acinetobacter gerneri* with dihydroxyacetone synthase (Das) from *Pichia angusta* in *E. coli*, which led to 22% of the carbon in phosphoenolpyruvate being derived from methanol[36].

In combination with pathway redesign, adaptive laboratory evolution (ALE) has been very effective in advancing synthetic methylotrophy. ALE harnesses the power of evolution to select mutations that benefit the desired phenotype, in this case production of cell biomass though the use of methanol[42]. A recent breakthrough with engineered *E. coli* assisted by ALE resulted in growth solely on methanol, with the shortest reported doubling time of 8.5 h (Fig. 2b). Intensive ALE delivered a methylotrophic *E. coli* strain with multiple copies of the heterologous methanol assimilation pathway genes replicated in its genome, the expression of which minimized cellular formaldehyde damage[13].

Although engineering of *E. coli* has been the main focus for synthetic methylotrophy, there have been parallel research achievements in other biotechnologically relevant microorganisms; *Corynebacterium glutamicum* and *Saccharomyces cerevisiae*. Similarly to the engineering in *E. coli*, a xylose-utilization pathway was constructed in *C. glutamicum* to generate a higher flux of the formaldehyde accepter Ru5P in an engineered strain expressing Mdh, Hps and Phi, with native formaldehyde dissimilation pathways blocked. Furthermore, *rpiB* encoding ribose phosphate isomerase B was deleted to prevent interconversion of Ru5P and R5P, and two rounds of ALE produced the best performing strain[43]. As a result, methanol and xylose were co-metabolized at an average mole ratio of 3.81:1. Synthetic methylotrophy attempts in *S. cerevisiae* have involved the expression of methanol assimilation pathways drawn either from the methylotrophic yeast, *P. pastoris*, the bacterial Mdh–Hps–Phi pathway, or a hybrid pathway with the bacterial Mdh and the yeast Das[44,45]. Recently, a weak native pathway for methanol assimilation was revealed in *S. cerevisiae*, which was further improved by ALE[45]. Interestingly, the assimilation route may involve dihydroxyacetone phosphate (DHAP), fructose 1,6-bisphosphate (F1,6BP) and G3P, similar to the XuMP pathway in *P. pastoris*. This finding challenges the previous understanding of methanol utilizers and is encouraging given the many important biotechnology processes carried out by yeast[45].

Methane as a C1 substrate

Methane, a potent GHG, is found extensively in nature, generated from both biogenic and anthropogenic sources such as biogas, and natural and shale gas. The abundance of methane, its low price and high degree of reduction make it an attractive substrate for biotechnology[46].

Methanotrophs can use methane as their sole carbon source under either aerobic or anaerobic conditions. The physiology and biochemistry of aerobic methanotrophs, in particular, have been thoroughly studied[47], and the first step of methane assimilation is the oxidation of methane to methanol, which is catalyzed by methane monooxygenases (MMOs) (Fig. 3b)[48]. Two types of MMOs are recognized: a membrane-bound, particulate enzyme complex, pMMO, is expressed in almost all methanotrophs, and the soluble form, sMMO, is present in a smaller range of methanotrophs. As methanol is formed, it is oxidized to formaldehyde which, in turn, can be dissimilated to CO_2 catalyzed by formaldehyde dehydrogenase (Ald) and formate dehydrogenase (Fdh)[47,49], or via the H_4F- or H_4MPT-mediated pathway[32].

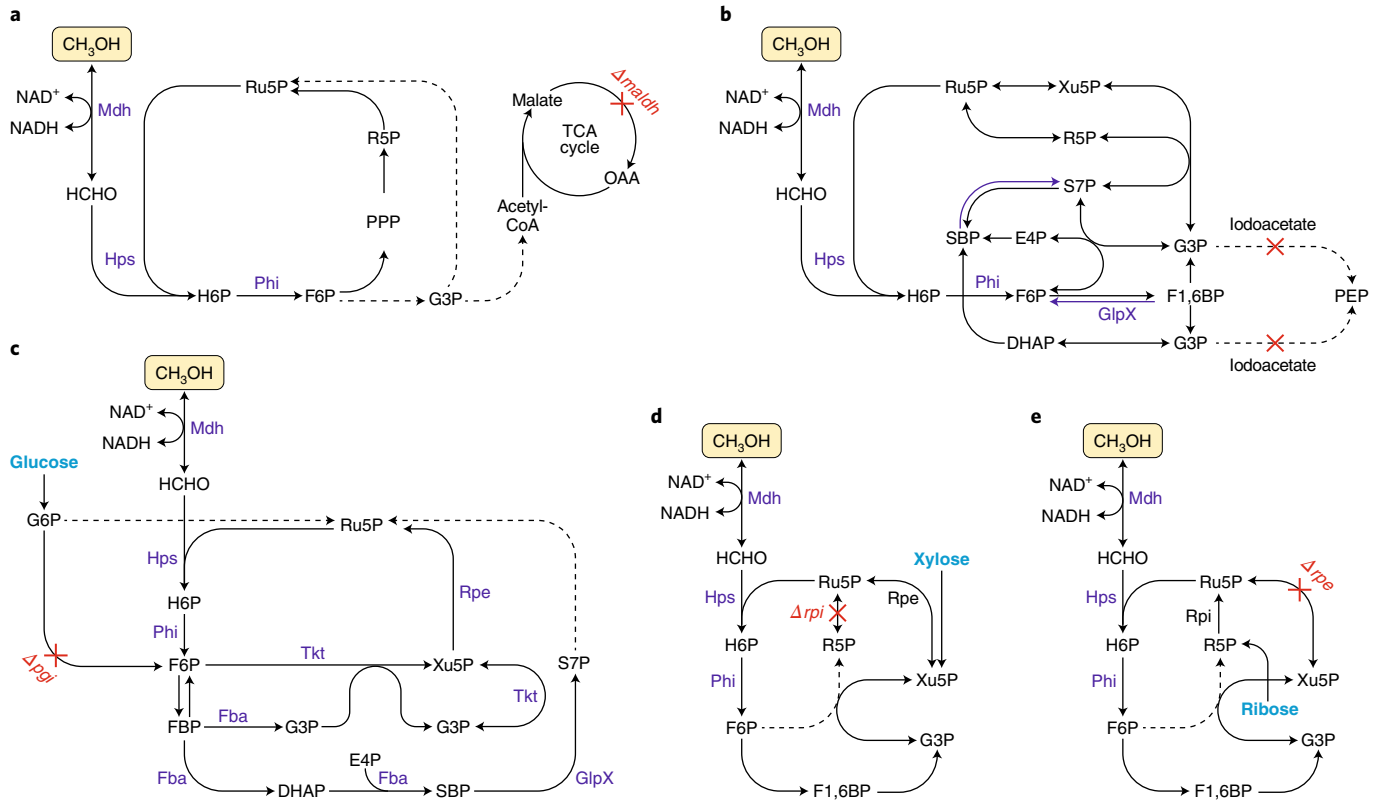

Fig. 4 | Typical metabolic engineering strategy applied to construction of synthetic methylotrophic *E. coli*. **a**, NAD^+ and NADH cofactor rebalancing strategy via the knockout of NAD^+-dependent malate dehydrogenase[40]. **b**, Strategy to improve formaldehyde consumption drives methanol assimilation through blockage of lower glycolysis[39]. **c**, Improving Ru5P cofactor availability through heterologous non-oxidative pentose phosphate pathway expression and phosphoglucose isomerase knockout[35]. **d**,**e**, Strategies to increase Ru5P availability to achieve synthetic methanol auxotrophy in *E. coli*[41]. Purple, heterologously expressed genes; red, deleted genes; blue, carbon sources other than methanol. Pathways blocked through gene deletions are shown by red crosses. Metabolites: H6P, F6P, F1,6BP, Ru5P, Xu5P, G3P, DHAP, DHA, 6PG, THF, E4P, S7P, R5P, sedoheptulose bisphosphate (SBP), PEP. Enzyme: Mdh, Hps, Phi, Pgi, FrmA, Pfk, Fba, Tkt, ribulose-phosphate 3-epimerase (Rpe), sedoheptulose bisphosphate (GlpX), fructose bisphosphatase (Fbp), ribose phosphate isomerase (Rpi).

Heterologous expression of MMOs with full activity has been a considerable challenge; for example, attempted biological methane oxidation with recombinant P450 monooxygenase has had low success[50,51]. Recently, however, expression of the β-subunit of pMMO has been achieved in *E. coli* with low methane oxidation activity[52], and catalytic pMMO domains from *Methylococcus capsulatus* have been expressed as soluble enzymes in *E. coli* and reassembled on an apoferritin particle scaffold, resulting in a pMMO-mimetic enzyme particle that exhibits similar methanol production kinetics in vitro to that of native pMMO[53].

Carbon monoxide as a C1 substrate

Carbon monoxide (CO) is a trace gas in the atmosphere, but an abundant waste gas in industrial processes[54]. Known for its toxicity, CO may impair oxygen transport and mitochondrial function in a wide range of organisms[55,56]. However, a phylogenetically diverse number of bacteria and archaea, referred to as carboxydotrophs[57], have evolved to use CO as a primary carbon and energy source[58]. These can be either aerobic or anaerobic microorganisms, and the latter can be further classified by the products that they make as hydrogenogens, acetogens, methanogens and sulfate-reducing bacteria[59,60].

The first reaction in CO assimilation is its oxidation to CO_2 by a carbon monoxide dehydrogenase (CODH) while providing reducing power to the cell[58]. Metal cofactors, molybdenum for aerobes and nickel for anaerobes, are essential to facilitate electron transport[61]. Aerobic carboxydotrophs typically use the Calvin–Benson–Bassham (CBB) cycle (described below) to yield biomass from the generated CO_2 (ref. [59]).

The Wood–Ljungdahl pathway (WLP)[62], also known as the reductive acetyl-CoA pathway, is the most widespread pathway among anaerobes. It consists of two linear branches to incorporate CO and CO_2 into biomolecules (Fig. 5a): the carbonyl branch, in which CO_2 is reduced to CO, and the methyl branch, in which CO_2 is converted into formate and further processed. The methylated corrinoid iron–sulfur protein product (CH3-COFeSP, Fig. 5a) is combined with a CO molecule to produce acetyl-CoA by the bifunctional enzyme CODH/acetyl-CoA synthase (ACS)[58]. The WLP is the signature pathway of acetogens, microorganisms that have attracted biotechnological interest and genetic and metabolic engineering reasearch[54].

Reconstructing the WLP in heterologous hosts is still a challenging aim for synthetic biology. The first attempts failed to demonstrate CODH/ACS activity in *E. coli* upon expression of five genes from *Morella thermoacetica*[63]. Although activity was later achieved after incubating ACS in a $NiCl_2$ solution[64], further efforts to reconstruct the entire pathway did not demonstrate growth using CO as a susbtrate[54]. Recent projects have used phylogenetically closer hosts and gene sources. The CODH and ACS proteins from *Clostridium carboxidovorans* were successfully expressed and formed a complex in *Clostridium acetobutylicum* but lacked in vivo activity[65]. In 2018, a set of genes from *Clostridium ljungdahlii*, encoding CODH/ACS,

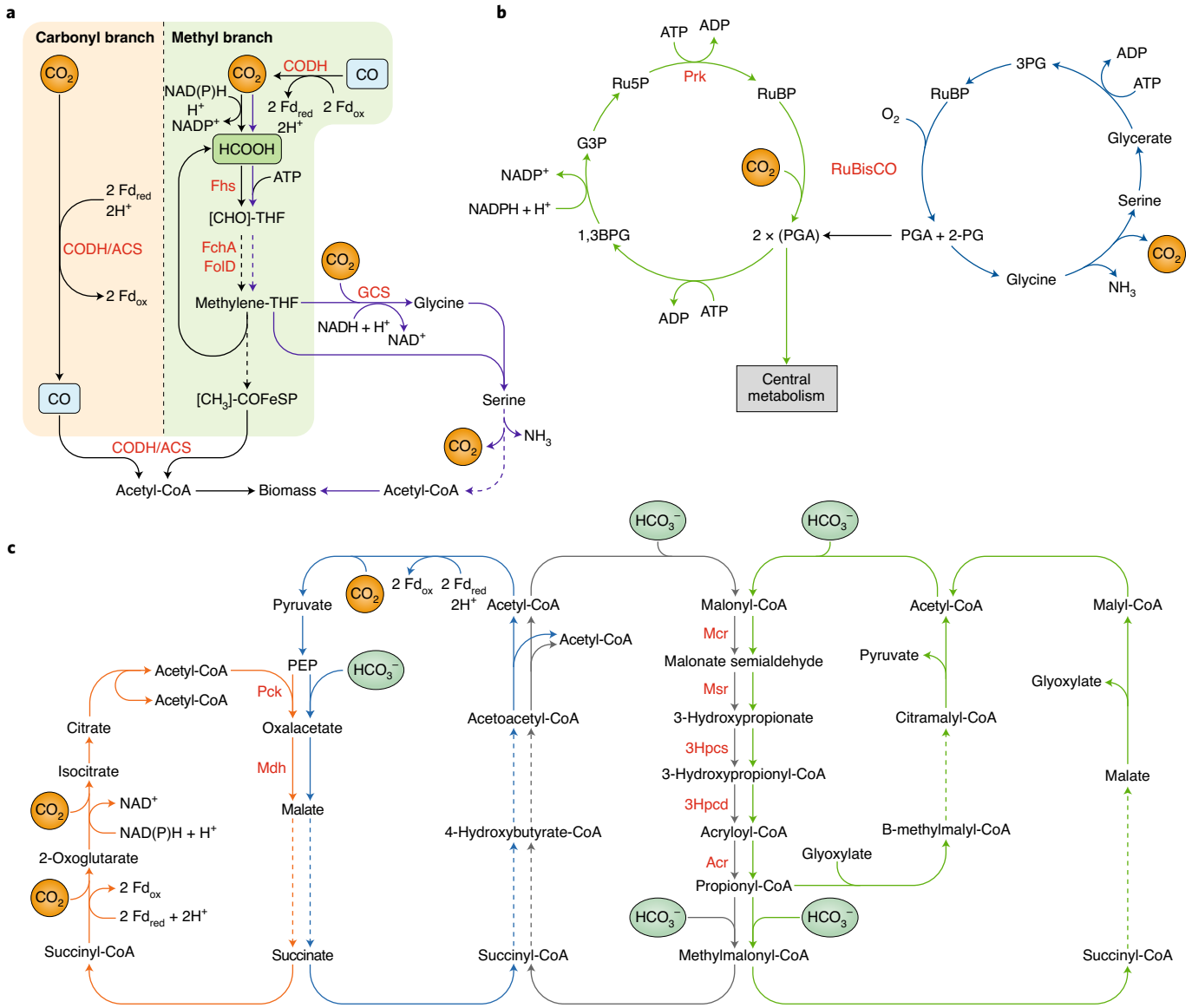

Fig. 5 | Natural CO_2 fixation pathways. All of the natural CO_2 fixation pathways (shown by different arrow colors). Genes that have been heterologously expressed are highlighted in red (except for the 3-HP bicycle, which was expressed entirely but broken down into submodules). **a**, The WLP (black arrows) and rGlyP (purple arrows) share a number of the methyl branch reactions until [CH3]-COFeSP is formed to continue the WLP and glycine to continue the rGlyP. **b**, The CBB cycle (left), responsible for CO_2 fixation via RuBisCO, and Its counter-cycle, photorespiration (right), in which RuBisCO uses oxygen instead of CO_2. **c**, The remaining CO_2 fixing pathways share a large number of intermediaries. These are (from left to right) the rTCA cycle (orange), DC/HB cycle (light blue), HP/HB cycle (brown) and 3-HP bicycle (green). Metabolites: THF, 10-formyltetrahydrofolate ([CHO]THF), methylated corrinoid iron-sulfur protein product (CH3-COFeSP), G3P, Ru5P, RuBP, 3PG, 1,3-diphosphoglycerate (1,3-BPG), 2-phosphoglycolate (2PG), PEP, 3-phosphoglycerate (PGA). Enzymes: RuBisCO, CODH, ACS, GCS, Pfk, malonyl-CoA reductase (mcr), malonate semialdehyde reductase (MSR), 3-hydroxypropionyl-CoA synthetase (3Hpcs), 3-hydroxypropionyl-CoA dehydratase (3Hpcd), acryloyl-CoA reductase (Acr), methylenetetrahydrofolate dehydrogenase (FolD), methenyltetrahydrofolate cyclohydrolase (FchA), formyltetrahydrofolate synthetase (Fhs), phosphoenolpyruvate carboxykinase (Pck), Mdh. Dashed arrows represent multiple reactions.

and a methylenetetrahydrofolate reductase from *E. coli* were introduced into *C. acetobutylicum*, reconstructing a functional WLP and demonstrating the fundamental role of the metal clusters[64]. Although these studies have proven successful, the complete transformation of non-acetogenic microorganisms into carboxydotrophs requires further effort.

Carbon dioxide as a C1 substrate

CO_2 represents 0.04% of Earth's atmosphere and is increasing on a global scale as the gas is released by human activities[66], and as such it is a major contributor to global warming. Autotrophic organisms are able to fix CO_2 from the environment and use it as a carbon source. Biotechnology offers the potential to not only capture CO_2 but also to transform it into valuable products, using either natural CO_2 fixing organisms (autotrophs)[67,68], or organisms engineered to acquire this ability (heterotrophs converted into chemoorganoautotrophs, hereafter referred as autotrophs)[11,12].

To date, several natural CO_2-fixing pathways have been described: the CBB cycle, the reductive TCA (rTCA) cycle, the dicarboxylate/4-hydroxybutyrate (DC/HB) cycle, the

3-hydroxypropionate-4-hydroxybutyrate (HP/HB) cycle, the 3-hydroxypropionate (3-HP) bicycle, the WLP and the reductive glycine pathway (rGlyP) (Fig. 5)[68,69]. The CBB cycle (Fig. 5b) accounts for 90% of global CO_2 fixation[70,71] and is present in both photosynthetic and non-photosynthetic organisms[71]. To fix CO_2, the cycle requires a single carboxylation reaction catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO)[72], which has low catalytic activity, low affinity for CO_2 and a preference for O_2. When performing its oxygenase activity that leads to photorespiration (Fig. 5b), CO_2 fixation is reduced by 50% and energy requirements increase[73–75]. Intensive research to attempt to improve the efficiency of RuBisCO towards CO_2 has had limited success[76,77].

Major efforts to transform heterotrophic microorganisms into CO_2 autotrophs have mainly focused on engineering the CBB cycle. Initial attempts to convert *E. coli* into CO_2 autotroph accomplished hemiautotrophic growth[78,79] whereby *E. coli* cellular metabolism was divided into separate modules and only partially relied on CO_2 as a carbon source. In the hemiautotrophic growth approach, one metabolic module fixed CO_2 to produce sugars and phosphorylated sugars through the expression of RuBisCO, phosphoribulokinase (Prk) and carbonic anhydrase (CA) enzymes, and the other module used organic energy carriers such as pyruvate, added exogenously to growth media, to produce energy and other cellular metabolites that originate from the TCA cycle[79] (Fig. 6a). In addition, to decouple the production of energy from carbon fixation, gluconeogenesis was blocked by deleting the phosphoglycerate mutase genes, *gpmA* and *gpmM*. The *aceBAK* operon was deleted to prevent the glyoxylate shunt, thus avoiding biomass growth from pyruvate utilizing the tartronate semialdehyde bypass. Although the first tests failed to demonstrate *E. coli* growth solely from CO_2 and pyruvate, the addition of a secondary carbon source, xylose, supported a productive CBB module. Selective pressure in xylose-limited chemostat evolution experiments, coupled to metabolic rewiring via deletion of phosphofructokinase A and B (PfkA and PfkB) and glucose -6-phosphate-1-dehydrogenase (Zwf) genes, resulted in sugar production from inorganic CO_2 (ref. [79]).

Complete CO_2 autotrophy was recently achieved in *E. coli* with formate added to generate reducing power, following the introduction of NAD^+-coupled Fdh from the methylotrophic bacterium *Pseudomonas* sp. 101 into the strain with some of the previously described genetic modifications (Figs. 2a and 6a)[12,79]. An ALE process, progressively decreasing the concentration of xylose in medium, was key to successful CO_2 autotrophy[12]. A similar approach was used for the transformation of the methylotrophic bacterium *Methylobacterium extorquens* AM1, in which RuBisCO and Prk were expressed to form an active CBB cycle, and its native methanol assimilation pathway was disrupted to utilize methanol as a source of energy and reducing power only[80]. However, here the approach failed to obtain consistent autotrophic growth, attributed to lack of balance between biosynthesis for biomass growth and for essential CO_2 fixation metabolites within the engineered strain[80]. By contrast, sustained growth solely from CO_2 was achieved in the methylotrophic yeast *P. pastoris* (Fig. 6b)[11,12]. Similarly to the aforementioned work[80], the use of methanol as a carbon source was blocked by deleting *DAS1*, *DAS2* and *AOX1* genes to reduce formaldehyde formation rate, while generating reducing power and energy through methanol's dissimilatory pathway supported by Aox2 (Fig. 6b). Alongside this, the enzymatic machinery to establish the CBB cycle was introduced into the peroxisome and ALE optimization led to increased growth rates on CO_2 (ref. [11]).

Prevention of the competing photorespiration activity of the CBB cycle in the presence of oxygen, and consequent loss of efficiency, can be achieved by maintaining high levels of CO_2 during culturing or through heterologous expression of carbon concentration mechanisms (CCMs). Carboxysomes, a type of CCM, are icosahedral proteinaceous structures that encapsulate RuBisCO and CA enzymes while concentrating CO_2 and HCO_3^- within the structure[81]. In one application of this concept, heterologously expressed carboxysomes enabled growth on CO_2 at atmospheric concentrations in an engineered *E. coli* strain expressing a total of 20 genes, including structural proteins and RuBisCO derived from *Halothiobacillus neapolitanus*, and Prk from *Synechococcus elongatus* PCC 7942 (ref. [81]).

Native CO_2-fixation pathways other than the CBB cycle have not been widely used from a metabolic engineering perspective to generate autotrophy in heterotrophs. However, the increasing availability of new tools for genetic modification and an improved understanding of cellular metabolism have the potential to reverse this. The rTCA cycle, identical to the TCA cycle but operating in the reverse (reductive) direction[67,71] (Fig. 5c), has been proposed for electrosynthesis processes[68], and production of high energy-cost products[82]. The rTCA pathway has been successfully engineered into *E. coli* and its expression targeted to the periplasm to achieve increased malate production, but CO_2 fixation was not considered in that study[83].

The DC/HB cycle (Fig. 5c) is an anaerobic pathway in which molecules of CO_2 and HCO_3^- are sequentially introduced into a cycle to form succinyl-CoA[67,71]. To our knowledge, this cycle has not been heterologously expressed. However, partial heterologous expression of the HP/HB cycle (Fig. 5c) from thermophile *Methallosphaera sedula* has been achieved in *Pyrococcus furiosus* and *E. coli*, using up to five genes to produce 3-hydroxypropionic acid and either acrylic or propionic acid[84,85], respectively. The 3-HP bicycle (Fig. 5c) is an energetically expensive pathway, half identical to the HP/HB cycle and comprising 19 reactions catalyzed by 13 different enzymes[71,73]. Recently, the 3-HP bicycle was divided into four modules, whose separate expression in *E. coli* showed correct activity paving the way for complete reconstruction of the pathway in heterotrophic hosts[82,86]. None of the individual modules were able to support autotrophic growth.

The WLP is the most energy efficient CO_2-fixation pathway, however only some of its reaction steps have been used to construct a synthetic rGlyP in microorganisms[14,87–90]. Although the WLP pathway is restricted to anaerobic conditions, the rGlyP can operate under aerobic conditions[14,82]. The rGlyP has been recently confirmed to exist in nature in *Desulfovibrio desulfuricans* G11 (ref. [69]) and is presented as an alternative for CO_2 fixation and formate assimilation (discussed below)[82]. It is noteworthy that fixation of CO_2 and formate was achieved in *E. coli* via a synthetic rGlyP pathway[87]. The employed electrochemical system also permitted formate to be generated via electrocatalysis from CO_2, meaning that CO_2 was the main feedstock for the microorganism[87].

Further CO_2-utilization synthetic pathways have been investigated for their potential to support autotrophy. The malonyl-CoA–oxaloacetate–glyoxylate (MOG) pathways replace RuBisCO with more efficient, oxygen tolerant enzymes such as phosphoenolpyruvate (PEP) carboxylase[75]. The CETCH cycle, which has been tested in vitro on the expression of 17 enzymes drawn from up to 9 different organisms, was completed with oxygen-insensitive and highly efficient enoyl-CoA carboxylases/reductases enabling 40% less energy consumption than the CBB cycle after an optimization process[70,82]. Finally, a new natural pathway, namely GED (Gnd–Entner–Doudoroff), was proposed and tested through flux models as the simplest pathway to achieve growth using CO_2. *E. coli* was engineered by specific gene deletions for reductive carboxylation with CO_2 of Ru5P, and following ALE improvements, demonstrated the in vivo function of the pathway and increased NADPH levels[91]. To conclude, recent progress in CO_2 fixation has been made by combining metabolic flux modelling, synthetic biology and ALE from both native and synthetic CO_2 fixation routes.

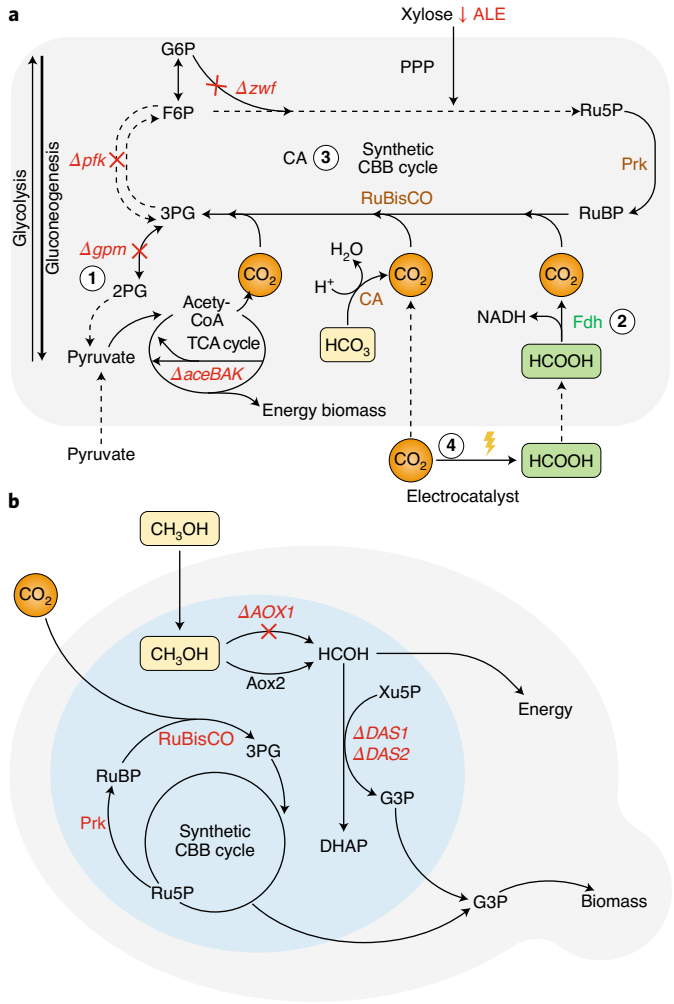

Fig. 6 | Engineered CO_2 utilization in *E. coli* and *P. pastoris*. **a**, Engineered *E. coli*, from current achievements to future implementations. The characteristic components of works (1) and (2) are shown in red and green, respectively, and the common elements are shown in brown. RuBisCO and Prk are introduced to create a synthetic CBB cycle (1). Biomass production is decoupled from energy generation by impairing gluconeogenesis (Δ*gpm*), leading to hemi-autotrophism[79]. Xylose and pyruvate are added externally to serve as the biomass precursor and to meet the new CBB cycle energy demands, respectively. The operon *aceBAK* is deleted to disable growth on pyruvate. The system is subjected to ALE limiting xylose input[79]. Fdh is then introduced to provide the cell with sufficient reducing power (2). The metabolic flux is redirected (Δ*pfk*, Δ*zwf*) towards the PPP and the new established CBB cycle. The system is further optimized via ALE, increasing organic carbon limitation[12]. Ultimately, RuBisCO can be encapsulated (3), enhancing its carboxylase activity through the introduction of CBs[81] and formate can be produced electrochemically from CO_2 (4)[87]. **b**, Engineered *P. pastoris*. Methanol metabolism is partially disrupted. Biomass generation from methanol is blocked (Δ*DAS1/DAS2*) and energy obtention via the dissimilatory branch is partially disrupted (Δ*AOX1*). A synthetic CBB cycle is introduced to recover the system via the heterologous expression of RuBisCO and Prk. The cycle is further compartmentalized in the peroxisome to obtain greater biomass yields[11]. Metabolites: glucose 6-phosphate (G6P), F6P, Ru5P, 3PG, RuBP, 2PG, Xu5P, G3P, DHAP. Enzymes: Fdh, Pfk, Prk, CA, RuBisCO and Aox2. The *aceBAK* operon encodes enzymes involved in the glyoxylate shunt.

Formate as a C1 substrate

Formate is attractive as a biotechnology substrate for its high water and polar solvent solubility (which facilitates storage and transport) and higher degree of reduction than CO_2 and CO. In addition, unlike methanol and methane, it is not flammable. Availability of formate is increasing due to developments in electrochemical, photochemical and catalytic methods for its production[92]. Also, as shown above, formate is an important intermediate in some CO_2 and methanol assimilation pathways.

There are two main strategies to utilize formate in microorganisms. The dissimilatory route involves the full oxidation of formate to CO_2, providing reducing power for carbon fixation, while the assimilatory route involves the condensation of formate with other metabolic intermediates[93]. Some of these pathways, such as the CBB cycle, the WLP, the RuMP cycle, the serine cycle and the rGlyP, have been described above and in recent reviews[31,93,94]. Based on existing and novel formate-fixing reactions, some proposed synthetic pathways could potentially support growth on formate[93–95]. However, these synthetic pathways are theoretical or have only been validated in vitro to date.

In terms of assimilatory pathways for formate, the rGlyP has been successfully introduced into *E. coli*[14,87,88], *S. cerevisiae*[89] and *Cupriavidus necator*[90] following a similar strategy as for the creation of glycine auxotrophic strains, where growth can be rescued if glycine is produced by C1 compounds such as CO_2, formate and methanol. In the first reported articles on *E. coli*, the upper pathway (shared with the WLP) that transforms formate into methylenetetrahydrofolate (methylene-THF) was completed, the glycine cleavage system (GCS) was introduced and endogenous genes involving glycine formation were overexpressed[87,88]. A similar approach using different gene variants achieved growth on formate and methanol[12,14]. In *S. cerevisiae*, constraining glycine metabolism to a single pathway and expressing endogenous genes supported CO_2 fixation and formate assimilation[89]. In *C. necator*, replacement of the natural CBB cycle by a synthetic rGlyP accomplished formatotrophic growth and CO_2 fixation[90]. Furthermore, ALE shortened the doubling time of engineered *E. coli*[14] (Fig. 2c) and *C. necator*[90].

In parallel, a reconstructed THF cycle allowed formic acid and CO_2 assimilation in *E. coli*[96,97]. This was achieved by expressing heterologous genes from *M. extorquens* to convert formate into methylene-THF and a reversed GCS achieved by deleting the repressor *gcvR*[96]. To further improve the pathway efficiency, a Fdh from *Candida boidinii* was expressed and reduced glucose usage, metabolic fluxes were adapted to augment gluconeogenesis and provide NADPH, and plasmid gene expression levels were tailored to optimize the levels of cytochrome bo3 and bd-I ubiquinol oxidase. The final engineered strain was able to grow on CO_2 and formate alone[97]. Although promising progress has been made in formate assimilation, low cell density and specific growth rate need further improvement[97].

Current challenges and future perspectives

C1 compounds can be naturally abundant, low-cost industrial by-products, or waste products that are widely available for use. Hence, exploitation of C1 compounds as alternative feedstocks for biomanufacturing can improve sustainability through the environmental benefits of reduced GHG emissions and reduced costs of production[1]. Engineering of C1-utilizing pathways into high-performing biotechnology hosts combines the productivity of industrial microorganisms that can take full advantage of C1 compounds as feedstocks. Advances in synthetic biology, metabolic engineering, and ALE have been key to developing synthetic C1-utilizing microbes (Supplementary Table 1). Although a series of achievements have been obtained in this field, there are still challenges remaining.

Importation of synthetic C1-utilization pathways into heterologous strains causes metabolite imbalance and the challenge remains as to how best to integrate heterogeneous C1-utilization pathways into endogenous metabolic pathways for effective C1 metabolism

and growth. An alternative strategy to address this challenge has been presented for CO_2 fixation by blocking pathways through gene deletion and reversing the common direction of enzyme-catalyzed reactions rather than introducing new pathways[91]. Another important issue is energy or cofactor imbalance resulting from forward reactions with C1 compounds. For example, the reducing power of NADH is insufficient to drive CO_2 fixation[12], and the high intracellular ratio of NADH to NAD^+ generated by the oxidation of methanol inhibits further oxidation of the substrate[39].

Deficiency of cellular resources such as acceptors (co-metabolites with C1s), cofactors (to assist C1 metabolism) and some enzymes (involved in C1 metabolism) constrains the effective use of C1 as carbon sources in engineered strains[35,39,53,61]. For example, in methanol utilization, rapid formaldehyde assimilation is required to both increase carbon flux into the cell and minimize its toxicity; this is chiefly controlled by the timely production of formaldehyde acceptor, Ru5P[5]. In addition, due to the low catalytic activity of RubisCO, high enzyme concentrations are required to catalyze the carboxylation reaction of the CBB cycle. Furthermore, the CBB cycle also consumes considerable ATP and NADPH to drive metabolism[71]. Synthetic methanotrophy has been constrained by the lack of functional expression of MMOs in industrial microorganisms. Also, the expression of metal cluster-containing enzymes involving in the DC/HB cycle for CO_2 fixation in heterogeneous organisms is a challenge due to incompatible intracellular conditions[61].

The toxicity of some C1s and intermediates generated during their metabolism needs careful management. In particular, the accumulation of formaldehyde as the initial step of methanol utilization causes toxicity to cells via the formation of crosslinks between and within proteins and DNA[24]. Although the toxic mechanisms of these C1 compounds is often understood, managing the flux of toxic substrates or intermediates within the cells to avoid levels above a threshold of harm is still challenging. At present, although several synthetic C1-utilizing microorganisms have been able to grow solely on C1s or on C1s with some auxiliary carbon source, their growth rate is well below their growth rate on sugars[11–14,35,36,41,43,79]. For example, the doubling time of wild-type *E. coli* on sugar-containing feedstocks is approximately 20 min, whereas the most advanced synthetic methylotrophic *E. coli* growing solely on methanol has a doubling time of 8.5 h (ref. [13]), and autotrophic *E. coli* utilizing CO_2 as a sole carbon source have a doubling time of 18 ± 4 h (ref. [12]).

To solve some of these issues, ALE combined with inverse metabolic engineering has been a powerful strategy to improve the performance of engineered organisms and to assess the impact of the radical metabolic changes brought about by synthetic C1 engineering[11–14,42]. Advantageous mutations generated during ALE enable metabolically engineered strains to adapt to imposed C1 metabolic pathways and improve the cells' tolerance to toxic C1 substrates, cofactor imbalances or intermediates generated in the process of C1 metabolism[12,13]. Slow growth from organisms engineered for C1 metabolism is the optimal screening pressure[98], and the effectiveness of screening for increased growth has been demonstrated by recent advances in synthetic CO_2 and methanol-utilizing organisms[12,13]. ALE has the potential to overcome intractable bottlenecks in the pathways, balance the expression of enzymes, enhance or inhibit catalytic activity, and assist our understanding of C1 metabolism.

New and developing technologies can help to improve research outcomes in C1 pathway engineering. Rational metabolic engineering is expected to further improve C1 use in heterologous hosts, and the benefits of computational approaches for metabolic pathway design and enzyme engineering have been evident in many of the advances in synthetic C1 utilization so far. In a complementary manner, the recent development of synthetic biology tools, such as combinatorial libraries of DNA parts, allows greater opportunity to control enzyme expression, improving the use of resources and reducing the accumulation of intermediates. In addition, dynamic control mechanisms based on engineered feedback regulation enable more balanced metabolic fluxes. Compartmentalization of C1 metabolic pathways into cellular organelles or the bacterial periplasm helps to avoid undesirable crosstalk with central metabolism or to prevent toxic metabolites entering the cytoplasm. Finally, construction of synthetic microbial communities in which one member of the community is a natural C1 utilizer that shares carbon with heterotrophs, can take advantage of C1 biotechnological processes to produce commodity and specialty chemicals[99].

Although C1 compounds may be toxic or produce metabolites that are toxic to industrial microorganisms, exploration of non-conventional organisms that have better tolerance towards these toxicants is worth considering. However, exploiting more suitable heterologous hosts or native organisms that use C1 as a carbon source will require the development of more advanced synthetic biology tools for these organisms than are currently available. Also, industry familiarity with these lesser known microorganisms in terms of performance and robustness will need to increase.

Looking beyond efficient C1 utilization, the future outlook is the development of C1-based biomanufacturing, in which synthetic C1-utilizer microbes are mature biotechnological platforms for converting C1 compounds to fuel and chemical products with high value[100]. As proof of concept, some microorganisms have been engineered to metabolize C1s to useful products such as methanol feedstock for the production of the flavonoid naringenin by engineered *E. coli*[34], and engineered *C. glutamicum* using this substrate plus xylose to form the amino acid glutamate[43].

Although constructing synthetic C1-utilizing organisms has been more challenging than expected, this field opens up a promising opportunity to shift the feedstock of biomanufacturing from a sugar-based carbon source to a sustainable, abundant non-food carbon source with low cost.

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Competing interests

The authors declare no competing interests.

Additional information

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