
Synthetic Methanol and Formate Assimilation Via Modular Engineering and Selection Strategies

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Abstract

One-carbon (C1) feedstocks can provide a vital link between cheap and sustainable abiotic resources and microbial bioproduction. Soluble C1 substrates – methanol and formate – could prove to be more suitable than gaseous feedstocks as they avoid mass transfer barriers. However, microorganisms that naturally assimilate methanol and formate are limited by a narrow product spectrum and a restricted genetic toolbox. Engineering biotechnological organisms to assimilate these soluble C1 substrates has therefore become an attractive goal. Here, we discuss the use of a step-wise, modular engineering approach for the implementation of C1 assimilation pathways. In this strategy, pathways are divided into metabolic modules, the activities of which are selected for in dedicated gene-deletion strains whose growth directly depends on module activity. This provides an easy way to identify and resolve metabolic barriers hampering pathway performance. Optimization of gene expression levels and adaptive laboratory evolution can be used to establish the desired activity if direct selection fails. We exemplify this approach using several pathways, focusing especially on the ribulose monophosphate cycle for methanol assimilation and the reductive glycine pathway for formate assimilation. We argue that such modular engineering and selection strategies will prove essential for rewiring microbial metabolism towards new growth phenotypes and sustainable bioproduction.

Introduction

One-carbon (C1) compounds could prove to be a crucial link between the abiotic and the biotic worlds. These feedstocks can be obtained from low-cost and abundant sources, such as syngas and natural gas (Dürre and Eikmanns, 2015; Clomburg *et al.*, 2017), and can be produced directly from CO₂ using energy sources such as sunlight and renewable electricity (Kumar *et al.*, 2012; Martín *et al.*, 2015; Claassens *et al.*, 2018; Jouny *et al.*, 2018). Multiple microorganisms can be cultivated on C1 compounds as sole carbon and energy sources, thus opening new avenues for sustainable bioproduction.

However, the use of microorganisms that can naturally grow on C1 substrates is limited by multiple factors, including a narrow product spectrum, low yields, titres, and productivities, a restricted genetic toolbox for engineering, and gaps in our understanding of their cellular physiology and metabolism (Whitaker *et al.*, 2015; Clomburg *et al.*, 2017). To overcome these difficulties, recent metabolic engineering efforts are aiming to introduce C1 assimilation pathways into model biotechnological microorganisms that are easier to engineer and that can be better optimized for industrially relevant conditions. These efforts use either natural pathways that are known to sustain high yields, or, more boldly, synthetic pathways with low ATP cost that could theoretically support increased yields (Bar-Even *et al.*, 2013; Siegel *et al.*, 2015; Bar-Even,

2016;). Some of these synthetic pathways can be established by combining naturally existing enzymes, while others include novel enzyme activities that can be realized by protein engineering (Erb *et al.*, 2017). In fact, engineered enzymes have already been demonstrated *in vitro* to support formate assimilation (Siegel *et al.*, 2015) and carbon fixation (Schwander *et al.*, 2016).

In this review, we discuss metabolic engineering studies aiming to introduce pathways for the assimilation of the soluble C1 compounds methanol and formate, the utilization of which bypasses the challenges associated with mass transfer of gaseous C1 substrates, such as methane and carbon monoxide (Henstra *et al.*, 2007; Fei *et al.*, 2014). We specifically focus on modular and selection-based engineering strategies in which the activity of pathway segments is coupled to cellular growth. We show that this step-wise approach is vital for the realization of synthetic C1 assimilation.

Modularity and selection as metabolic engineering strategies

Engineering synthetic C1 metabolism requires the overexpression of pathway enzymes, especially those that are missing in the host or that are natively expressed at insufficient levels. However, simple overexpression is unlikely to be sufficient for realizing the activity of the entire pathway. This is mainly because of the overlap between the introduced pathway and the host central metabolism, resulting in disrupted fluxes through both systems. To better identify and resolve problematic metabolic interactions, it is helpful to divide the synthetic pathway into smaller metabolic modules, i.e. sub-pathways consisting of several reactions (Fig. 14.1). The *in vivo* implementation of these modules can be considerably easier than the full pathway and provide vital information on the metabolic context that enables or constrains the newly introduced

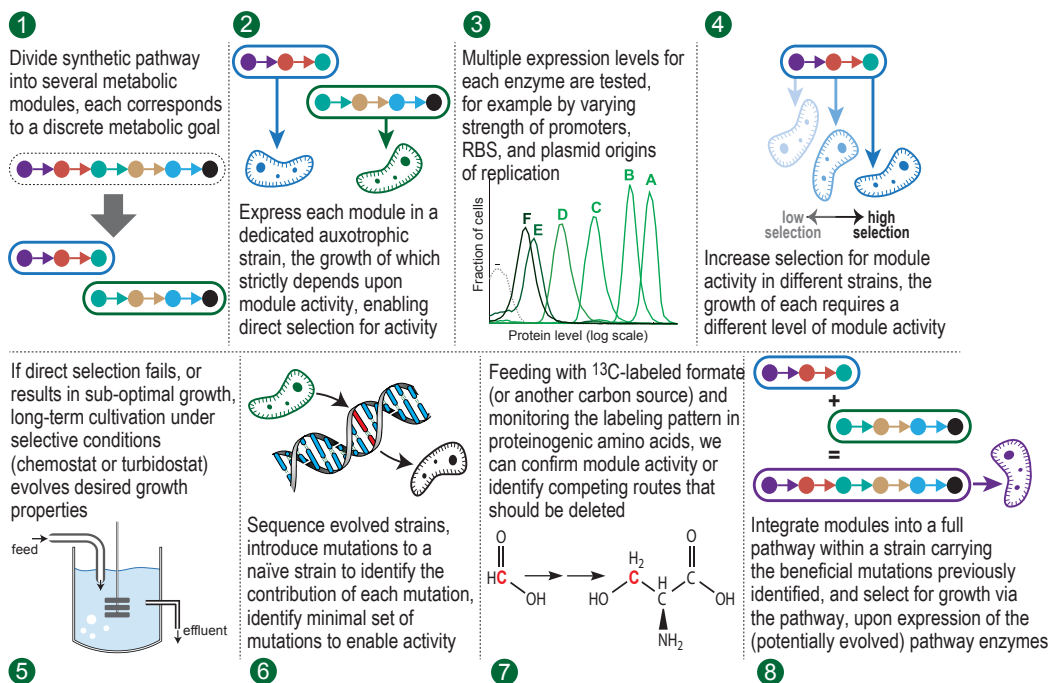


Figure 14.1 A schematic representation of the modular engineering and selection approach outlined in this paper.

activities. To probe the implementation of metabolic modules, it is useful to couple their activity with the growth of the host.

Coupling module activity with growth usually requires modifying the metabolic network of the host by performing strategic gene deletions. These are made to generate a strain auxotrophic for certain essential metabolic intermediates – for example, an amino acid – which can be exclusively synthesized via the synthetic module. As a result, cellular growth becomes dependent on the activity of the module. A range of selection strains can be designed with increasing selection pressure for pathway activity: a ‘minimal’ selection is sustained if the module provides a single required metabolite, higher selection pressure is obtained when module activity is responsible for the biosynthesis of multiple building blocks, and very high selection pressure is imposed when the biosynthesis of all or most biomass is dependent on the module.

The design of modules and selection strains can be assisted by computational tools based on Flux Balance Analysis, for example OptKnock or FlexFlux (Burgard *et al.*, 2003; Marmiesse *et al.*, 2015; Meyer *et al.*, 2018). Yet, in most cases, manual design based on biochemical and metabolic knowledge suffices. Specifically, when dividing a pathway into metabolic modules, several factors should be taken into consideration (Wenk *et al.*, 2018): (1) the module should be linked to a clear growth phenotype within an appropriate selection strain, resulting in growth versus no growth readout; (2) the number of enzymes in a module should be limited, to enable easy expression and optimization, and to allow straightforward interpretation of growth phenotypes; (3) modules should together cover the whole pathway and could overlap with one another, such that enzymes occurring in multiple modules can be tested in different metabolic contexts; (4) ideally, modules should be easy to combine, that is, dedicated selection strains – whose growth is dependent on the activity of several consecutive modules – should be easy to construct.

When direct selection for module activity fails or results in poor growth, further optimization is required (Fig. 14.1). This can be achieved by modulating the expression levels of the pathway enzymes (Zelcbuch *et al.*, 2013; Wenk *et al.*, 2018),

and potentially also of related host enzymes, e.g. deletion or down-regulation of enzymes that divert metabolic intermediates from the pathway. In addition, different enzyme variants or codon optimization of the relevant genes can support increased expression and activity. Another method, which does not rely on genetic tools, is the addition of small molecules that specifically inhibit interfering enzymes, as demonstrated for the glycolytic glyceraldehyde 3-phosphate dehydrogenase in the engineering of methanol assimilation in *Escherichia coli* (Woolston *et al.*, 2018a).

If these approaches fail to establish module activity, adaptive laboratory evolution (ALE) can be performed to increase module functionality and establish module-dependent growth (Portnoy *et al.*, 2011). For this process, the overexpressed genes should preferably be integrated into the genome rather than carried on a plasmid as to increase the chance of beneficial mutations to be fixed in the population. Different types of ALE can be applied; a prominent approach being continuous cultivation on a selective medium, with limiting amounts of the compounds for which the cells are auxotrophic. This method was applied for the successful engineering of the CO₂-fixing Calvin cycle in *E. coli* (Antonovsky *et al.*, 2016), where the concentration of the limiting substrate xylose was gradually decreased as growth improved. Another approach involves swapping between permissive and selection media, where the former contains the auxotrophy-related compounds and the latter does not (Marlière *et al.*, 2011). The relative dosages of the different media are coupled to the growth of the population, such that increased cell density leads to addition of selection medium, and a decrease in cell density results in more permissive medium. Such cultivation regime adapts the population towards using the selection medium until the permissive medium is no longer required (Marlière *et al.*, 2011; Bouzon *et al.*, 2017; Döring *et al.*, 2018). Following successful evolution of module activity, the evolved genomes can be sequenced to identify the accumulated mutations. The specific effect of different mutations can be interpreted and further studied by reintroducing them into a non-evolved selection strain.

Synthetic methanol assimilation

Methanol can be produced from diverse fossil and renewable sources and has been proposed as a promising feedstock for industrial applications and microbial growth (Olah *et al.*, 2009; Olah, 2013; Zhang *et al.*, 2017). Hence, biological conversion of methanol to products has received considerable attention (Schrader *et al.*, 2009; Whitaker *et al.*, 2015; Pfeifenschneider *et al.*, 2017; Bennett *et al.*, 2018a). As genetic toolboxes for the engineering of most promising natural methylotrophs (e.g. *Bacillus methanolicus*) are still underdeveloped, engineering model biotechnological microbes for growth on methanol has become an attractive target.

Three native pathways are known to support growth on methanol: the ribulose monophosphate (RuMP) cycle, the xylulose monophosphate cycle, and the serine pathway (Kato *et al.*, 2006; Chistoserdova *et al.*, 2009). Among these, the engineering of the RuMP cycle has received most attention, as this route supports the highest yield (Bar-Even *et al.*, 2013). The heterologous establishment of the serine pathway has not yet been reported and only a single study aimed at engineering the xylulose monophosphate pathway in *Saccharomyces cerevisiae* (Dai *et al.*, 2017).

A synthetic methanol assimilation pathway has also been proposed, where three formaldehyde molecules are condensed to dihydroxyacetone by a rationally engineered formolase enzyme. This pathway was recently introduced in *E. coli*, but did not lead to substantial methanol assimilation, probably due to the poor kinetics of the formolase enzyme (Wang *et al.*, 2017). Another proposed synthetic methanol condensation cycle, which was demonstrated *in vitro*, consists of the RuMP cycle combined with non-oxidative glycolysis (Bogorad *et al.*, 2014). In this pathway, fructose-6-phosphate (F6P) is cleaved by phosphoketolases to produce acetyl-CoA, bypassing pyruvate decarboxylation and preventing carbon loss. Finally, an alternative, synthetic structure of the serine pathway was proposed, in which serine is deaminated to pyruvate, and glycine is recycled via threonine biosynthesis and degradation, further generating acetyl-CoA as a biomass precursor (Bar-Even, 2016). A recent study describes the successful selection for key metabolic modules of this synthetic route, that is, formate assimilation into serine via the

tetrahydrofolate (THF) system and glycine production solely from threonine cleavage (Yishai *et al.*, 2017).

As the RuMP cycle (Fig. 14.2a) attracts the most research attention so far, we will focus on attempts for its engineering in biotechnological hosts. Non-methylotrophic hosts typically lack only three enzymes of the pathway: methanol dehydrogenase, hexulose-6-phosphate synthase and 6-phospho-3-hexulosiomerase. Several studies have overexpressed these enzymes in *E. coli* (Müller *et al.*, 2015; Price *et al.*, 2016; Whitaker *et al.*, 2017; Bennett *et al.*, 2018b; Gonzalez *et al.*, 2018) and *Corynebacterium glutamicum* (Leßmeier *et al.*, 2015; Witthoff *et al.*, 2015) and have demonstrated methanol assimilation, albeit at low rates and assimilation efficiencies. These efforts, however, did not apply a selection strategy, that is, cellular growth was not dependent on methanol assimilation.

The RuMP cycle can be divided into three main modules: methanol oxidation to formaldehyde, formaldehyde assimilation into central metabolism, and regeneration of the acceptor metabolite ribulose 5-phosphate (Ru5P) (Fig. 14.2b). In the assimilation module, formaldehyde is condensed with Ru5P to generate F6P, which can be metabolized to all biomass building blocks. The assimilation and regeneration modules can be supported by several alternative metabolic structures, the most efficient one uses glycolysis and the non-oxidative pentose phosphate pathway (Quayle and Ferenci, 1978; Zhang *et al.*, 2017).

Several recent studies have attempted direct selection for the activities of the methanol oxidation module and the formaldehyde assimilation module in *E. coli* (Chen *et al.*, 2018; Meyer *et al.*, 2018) and *C. glutamicum* (Tuyishime *et al.*, 2018). These studies generated a selection strain in which ribose 5-phosphate isomerase is deleted ($\Delta rpiAB$). This knockout blocked growth on xylose or gluconate (the latter with the additional deletion of *edd*, encoding phosphogluconate dehydratase). The methanol oxidation and formaldehyde assimilation modules were expected to rescue growth on xylose or gluconate by enabling their assimilation via a 'RuMP shunt', a linear route converting Ru5P to F6P via condensation with formaldehyde (Fig. 14.2c and d). Yet, none of these studies was able to demonstrate the required activity upon direct

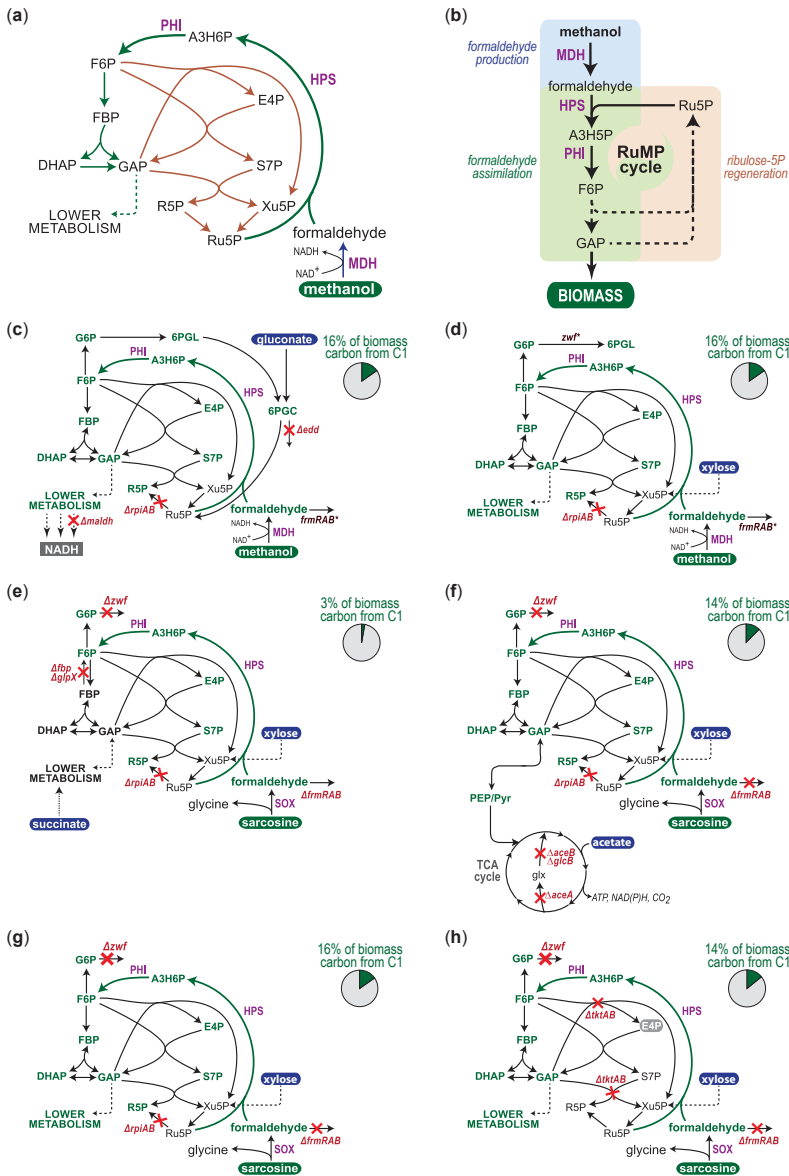


Figure 14.2 Engineering the RuMP shunt in *E. coli*. (a) Metabolic structure of a variant of the RuMP cycle. (b) Subdivision of the RuMP cycle into metabolic modules; (c, d) Selection schemes for methanol-dependent growth of *E. coli* via the RuMP shunt as described by Meyer *et al.* (2018) (c) and Chen *et al.* (2018) (d). (e–h) Selection schemes for growth via the RuMP shunt at different selection strengths as described in He *et al.* (2018). Pie charts indicate the minimum fraction of carbons in biomass that are derived from C1 (formaldehyde or methanol) in the different selection schemes, as calculated using (Neidhardt *et al.*, 1990). Gene deletions are shown in red; overexpressed enzymes in purple; enzymes that mutated during ALE in brown; metabolites that are dependent on the RuMP shunt in green; and substrates that have to be co-fed in blue. Enzyme abbreviations: aceA, isocitrate lyase; aceB, malate synthase B; edd, phosphogluconate dehydratase; fbp; fructose-1,6-bisphosphatase; frmRAB, glutathione-dependent formaldehyde detoxification system; glcB, malate synthase G; glpX, fructose-1,6-bisphosphatase 2; HPS, 3-hexulose-6-phosphate synthase; maldh, malate dehydrogenase; MDH, methanol dehydrogenase; PHI, 6-phospho-3-hexuloisomerase; rpiAB, ribose 5-phosphate isomerase; SOX, sarcosine oxidase; tktAB, transketolase A and B; zwf, glucose 6-phosphate dehydrogenase. Metabolite abbreviations: A3H6P, arabino 3-hexulose 6-phosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose bisphosphate; GAP, glyceraldehyde 3-phosphate; 6PGC, 6-phospho-gluconate; 6PGL, 6-phospho-glucono-lactone; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; Xu5P, xylulose 5-phosphate.

selection, and ALE was necessary to establish methanol-assimilation-dependent growth.

The strict requirement for ALE to achieve RuMP shunt-dependent growth can be attributed to the unfavorability of methanol oxidation. Supporting this notion, all of these studies found mutations linked to a decreased NADH/NAD ratio, e.g. by interrupting or inhibiting the TCA cycle (Chen *et al.*, 2018; Meyer *et al.*, 2018; Tuyishime *et al.*, 2018). This likely relates to the fact that methanol oxidation is thermodynamically limited by a high NADH/NAD ratio. Using deuterated methanol (CD_3OD), it was shown that methanol dehydrogenase also kinetically limits the activity of the RuMP shunt in *E. coli* (Woolston *et al.*, 2018a). Methanol oxidation could be improved by identifying or engineering kinetically superior variants, which could be directly screened using a formaldehyde biosensor (Woolston *et al.*, 2018b) or selected for in appropriate selection strains.

To overcome the barriers associated with methanol oxidation, we decided to separate the formaldehyde production module from that of formaldehyde assimilation, such that we could test the latter in more detail. Towards this aim, we replaced methanol dehydrogenase with the kinetically efficient and thermodynamically favourable sarcosine oxidase that metabolizes sarcosine to formaldehyde and glycine (He *et al.*, 2018). We further constructed several selection strains, imposing different levels of selective pressure on formaldehyde assimilation via the RuMP shunt. These included (i) $\Delta\text{frmRAB } \Delta\text{rpiAB } \Delta\text{fbp } \Delta\text{glpX } \Delta\text{zwf}$ strain in which the synthetic shunt provides only essential sugar phosphates while succinate serves as the main growth substrate (Fig. 14.2e); (ii) $\Delta\text{frmRAB } \Delta\text{rpiAB } \Delta\text{aceBA } \Delta\text{glcB } \Delta\text{zwf}$ strain in which the RuMP shunt provides almost all cellular building blocks while acetate oxidation provides reducing power and energy (Fig. 14.2f); (iii) $\Delta\text{frmRAB } \Delta\text{rpiAB } \Delta\text{zwf}$ strain, where the shunt is responsible for satisfying all cellular carbon and energy needs (Fig. 14.2g); and (iv) $\Delta\text{frmRAB } \Delta\text{tktAB } \Delta\text{zwf}$ strain in which almost all cellular carbon and energy needs are supported by the RuMP shunt (Fig. 14.2h).

We were able to directly select for growth of all of these strains via the RuMP shunt without the need for ALE (He *et al.*, 2018), most probably because our selection scheme bypasses the challenge of methanol oxidation. However, the growth

we obtained with the $\Delta\text{frmRAB } \Delta\text{rpiAB } \Delta\text{zwf}$ strain was considerably poorer than that observed using the $\Delta\text{frmRAB } \Delta\text{tktAB } \Delta\text{zwf}$ strain. This serves as a clear demonstration of the importance of the metabolic context for establishing activity of a newly introduced pathway. In this specific case, the poor growth associated with the $\Delta\text{frmRAB } \Delta\text{rpiAB } \Delta\text{zwf}$ strain probably stems from costly metabolism of F6P ‘back’ to xylulose-5-phosphate and Ru5P, and potentially from the inhibition of essential transketolase reactions due to the accumulation of the coproduct xylulose-5-phosphate (He *et al.*, 2018).

The major challenge of establishing a fully functional RuMP cycle lies in the regeneration module. To make things even more difficult, the establishment of autocatalytic cycles, such as the RuMP cycle, requires the kinetic parameters of enzymes to be carefully balanced as to avoid draining the pathway intermediates (Barenholz *et al.*, 2017). A recent study suggested an interesting way to tackle this challenge, by overexpressing the irreversible SBPase to force flux towards the regeneration of Ru5P (Woolston *et al.*, 2018a). Another approach is to overexpress the enzymes of the pentose phosphate pathway from an organism that supports more efficient regeneration of Ru5P (Bennett *et al.*, 2018b). Still, as was previously shown with ALE of *E. coli* to achieve a functional Calvin Cycle, down-regulation of branching enzymes, e.g. ribose-phosphate diphosphokinase, might be necessary to establish a sustainable cyclic flux (Antonovsky *et al.*, 2017; Herz *et al.*, 2017).

Synthetic formate assimilation

Formate is a promising microbial feedstock that can be efficiently produced from CO_2 by electrochemical and photochemical processes (Kopljár *et al.*, 2016; Zhou *et al.*, 2016; Yang *et al.*, 2017). Formate can also be obtained from partial oxidation of biomass or natural gas and by hydrogenation of CO_2 (Shen *et al.*, 2015; Wang *et al.*, 2015).

Microbial growth on formate as a carbon and energy source is reported for diverse groups of microorganisms. Acetogenic and methanogenic microbes can grow on formate using the reductive acetyl-CoA pathway (Kerby and Zeikus, 1987), representing the most efficient way to convert this feedstock into a product (Bar-Even *et al.*, 2013).

However, this anaerobic pathway can support only a narrow product spectrum (Bertsch and Müller, 2015). Aerobic growth on formate is sustained by the Calvin cycle and the serine pathway; however, the high ATP costs of these pathways reduce the potential biomass and product yields (Bar-Even *et al.*, 2013). To overcome this problem, several synthetic pathways have been designed to support high yields under aerobic conditions (Bar-Even *et al.*, 2013; Siegel *et al.*, 2015; Bar-Even, 2016).

In the synthetic formolase pathway, formate is first reduced to formaldehyde by promiscuous activity of two enzymes: acetyl-CoA synthase and acetylaldehyde dehydrogenase (Siegel *et al.*, 2015). Then, three formaldehyde molecules are condensed into dihydroxyacetone by the formolase enzyme described before. The activity of the pathway was demonstrated *in vitro*, but its *in vivo* functionality was very poor due to the low activities of formaldehyde dehydrogenase and the formolase enzyme (Siegel *et al.*, 2015). To boost the activities of these limiting enzymes, a modular selection strategy can be used. For example, a module responsible for formate reduction to formaldehyde can be tested in a formaldehyde-dependent strain – for example, the RuMP shunt-dependent strains described above – and ALE can be used to increase this activity. Similarly, activity of the formolase enzyme might be initially tested and optimized in a strain which produces formaldehyde via sarcosine oxidation and assimilate dihydroxyacetone to provide only a fraction of cellular building blocks. For example, by deleting phosphoglycerate mutase, central metabolism can be divided into upper and lower segments, where succinate provides carbon and energy for lower metabolism and dihydroxyacetone phosphorylation provides carbon only for upper metabolism (Zelcbuch *et al.*, 2015). This would impose moderate selection for the activity of the formolase enzyme and would thus be a more reasonable initial target.

A more feasible approach to establish formate assimilation would be to focus on existing enzymatic activities that could be combined to realize a new pathway. This is exactly the case of the synthetic reductive glycine pathway (Fig. 14.3a) (Bar-Even *et al.*, 2013). All the reactions of this pathway are catalysed by known and ubiquitous enzymes. It is even possible that the pathway operates endogenously in some microbes (Figueroa

et al., 2017). Its limited overlap with central metabolism and its very high ATP-efficiency further make the reductive glycine pathway especially promising to support aerobic formate assimilation.

To facilitate the implementation of the reductive glycine pathway in *E. coli*, we divided it into four modules (Fig. 14.3a): (i) a C1 activation module in which formate is condensed with the THF system and reduced to 5,10-methylene-THF; (ii) a glycine biosynthesis module that condenses the C1 moiety from 5,10-methylene-THF with CO₂ and ammonia to generate the C2 metabolite glycine; (iii) a serine biosynthesis module that condenses glycine with another C1 moiety to give the C3 metabolite serine; and (iv) a serine assimilation module that deaminates this amino acid to produce pyruvate as a biomass precursor.

We constructed several selection strains to demonstrate the activities of the pathway modules in *E. coli* (Yishai *et al.*, 2017, 2018). First, we constructed a C1-auxotroph strain ($\Delta glyA \Delta gcvTHP$) and showed that overexpression of formate THF ligase (FTL) enabled formate to serve as sole source of all C1-dependent building blocks, including purines, thymidine, and methionine (Fig. 14.3b). This confirmed the efficient activity of the C1-activation module. Next, we selected for the combined activity of the C1-activation module and the serine biosynthesis module. Towards this aim, we constructed a strain deleted in 3-phosphoglycerate dehydrogenase ($\Delta serA$, the first enzyme of serine biosynthesis) and the glycine cleavage system ($\Delta gcvTHP$). Only upon overexpression of FTL and the native bifunctional 5,10-methenyl-THF cyclohydrolase/5,10-methylene-THF dehydrogenase (FolD), glycine and formate could serve as C1 and serine sources to support cell growth (Fig. 14.3c). This demonstrates that testing a module – in this case the C1 activation module – in different metabolic contexts is important to uncover hidden bottlenecks: while the endogenous activity of FolD sufficed for the first selection strain, the higher activity required for the growth of the second selection strain necessitated dedicated overexpression of the enzyme (Yishai *et al.*, 2017).

In a follow-up study, we selected for the activities of the C1-assimilation module and the glycine biosynthesis module (Yishai *et al.*, 2018). We constructed a strain auxotrophic to C1 and glycine ($\Delta glyA \Delta ltaE \Delta kbl \Delta aceA$). We could establish

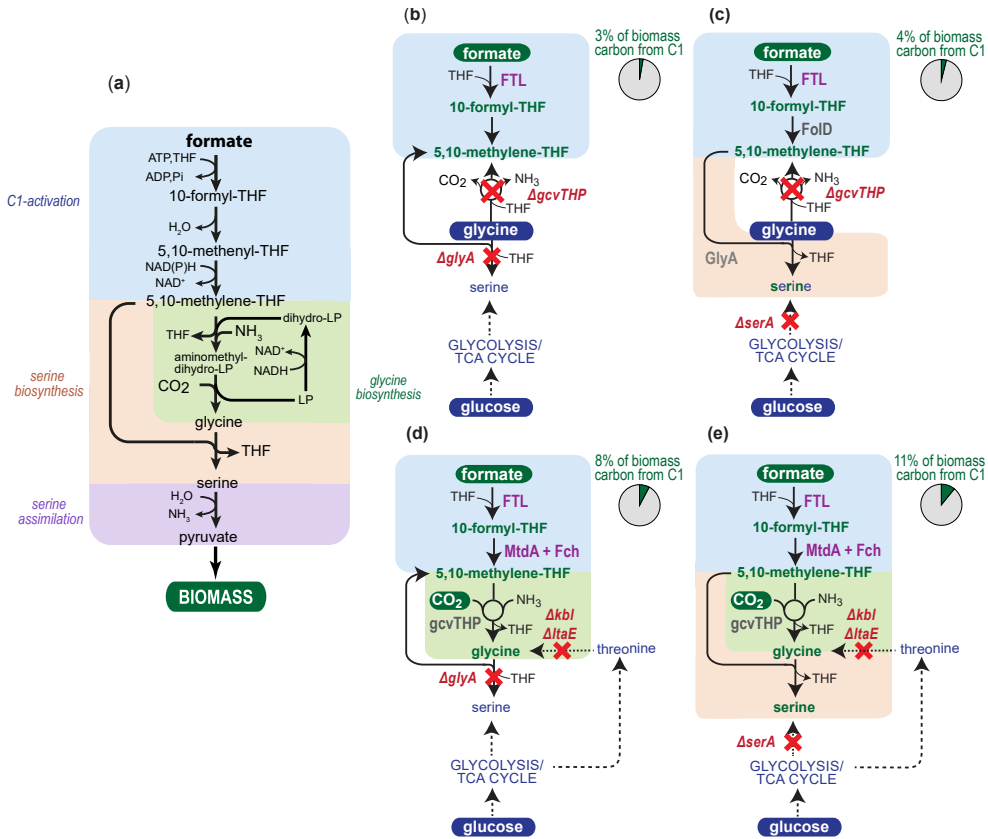


Figure 14.3 Engineering the reductive glycine pathway in *E. coli*. (a) Metabolic scheme of the reductive glycine pathway and its subdivision into modules; (b–e) Selection schemes for the activity of different modules of the reductive glycine pathway, as demonstrated in *E. coli* (Yishai *et al.*, 2017, 2018). Pie charts indicate the minimum fraction of carbons in biomass that are derived from formate and CO₂ in the different selection schemes, as calculated using (Neidhardt *et al.*, 1990). Gene deletions are shown in red; overexpressed foreign enzymes in purple; overexpressed native enzymes in grey; metabolites that are produced from formate and CO₂ in green; and substrates that have to be co-fed in blue. Abbreviations: Fch, 5,10-methenyl-THF cyclohydrolase; FOL, bifunctional 5,10-methenyl-THF cyclohydrolase/5,10-methylene-THF dehydrogenase; FTL, formate-THF ligase; gcvT_{HP}(L), glycine cleavage system subunits T, H and P (and lipoamide dehydrogenase subunit); glyA, serine hydroxymethyltransferase; ltaE, threonine aldolase; kbl, 2-amino-3-ketobutyrate CoA ligase; LP, lipoamide-protein; MtdA, 5,10-methylene-THF dehydrogenase; serA, 3-phosphoglycerate dehydrogenase; THF, tetrahydrofolate.

formate conversion to C1-activated-THF and glycine upon overexpression of the enzymes of the native glycine cleavage system as well as FTL, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase from *Methylobacterium extorquens* (Fig. 14.3d). The latter two enzymes were necessary to replace the native FOL, whose activity was too low to support the required flux even when overexpressed, probably since it is inhibited by the key intermediate 10-formyl-THF (Yishai *et al.*, 2018). Next, to select for the activity of three of the pathway modules, we constructed

a C1-glycine-serine auxotrophic strain (Δ serA Δ ltaE Δ kbl Δ aceA) (Fig. 14.3e). Overexpression of enzymes mentioned above resulted in C1, glycine and serine production solely from formate and CO₂. Overall, \approx 10% of carbons in biomass were provided by the pathway and the fast growth obtained (doubling rate of \approx 1.7 hours) indicates high activity of all pathway components.

Another recent study also focused on modular engineering of the reductive glycine pathway in *E. coli* (Tashiro *et al.*, 2018). Similar to the approach described above, this study demonstrated that

the overexpression of three foreign genes from *Clostridium ljungdahlii* can support C1 and serine biosynthesis from formate and glycine (Fig. 14.3c). However, for demonstration of glycine and serine biosynthesis via the reductive activity of the glycine cleavage system, leaky auxotrophic strains were employed in which only *serA* or *glyA* were deleted, while threonine cleavage to glycine (via Kbl or LtaE) was left untouched. Consequently, as is supported by the labelling data, in these strains, the majority of glycine and serine were most probably derived from threonine cleavage rather than formate assimilation.

In a parallel effort to the rational engineering approach, we demonstrated the establishment of the three modules of the reductive glycine pathway using ALE (Döring *et al.*, 2018). This work used the same tight selection strategies as above but introduced only a single foreign enzyme: *Clostridial* FTL that was integrated into the genome. The cells were cultivated continuously and provided with two types of alternating media: a selection medium (which does not contain the auxotrophy-relieving compounds) that was added when the culture turbidity surpassed a threshold, and a permissive medium (containing the compounds for which the strains are auxotrophic) that was supplied upon decrease of the culture turbidity below this threshold. This procedure was used to select for growth on the stressing medium and, using sequential rounds of evolution, established metabolism of formate to C1-activated-THF, glycine, and, finally, serine. Genome sequencing of these strains revealed several mutations, including in the coding region of the key limiting enzyme FolD. Several genes were duplicated in the genome, including FTL and FolD, probably to increase their expression levels. This study confirms that the same metabolic goals can be achieved either via a rational engineering approach or via long term evolution.

To complete the establishment of the reductive glycine pathway, the next challenge is to integrate the first three modules – converting formate to serine – with the last one, that is, serine assimilation into central metabolism. This would require an order of magnitude increase in the flux via the pathway and would most probably require ALE to adapt the cellular physiology for the novel growth mode.

Conclusions

Recent efforts using modular and selection-based engineering approach have enabled C1-routes to support the biosynthesis of a substantial fraction of the host's biomass. These studies demonstrate a general approach of iterative design, build, test and learn cycles. First, pathway modules and selection strains are designed and constructed. Testing module activity using these selection strains provides insights into the metabolic constraints that limit flux, from which better designs and improved activity can emerge. Selection strains are therefore an essential tool for these cycles, as they provide simple performance readout that facilitates optimization of module and pathway activity.

As mentioned above for the RuMP cycle, a major challenge for establishing C1 pathways relates to the regulation of flux that is diverted away from the pathway towards other biosynthesis routes. This is especially true for autocatalytic cycles in which the product is also an intermediate of the pathway. To realize stable activity, proper balancing is required between the rates of pathway enzymes and those of the branching enzymes. As previously demonstrated, for autocatalytic cycles, this likely requires ensuring low affinities of the branching enzymes towards the pathway metabolites (Barenholz *et al.*, 2017). Indeed, the use of ALE for the establishment of a functional Calvin cycle in *E. coli* resulted in lower activity of branching reactions (Antonovsky *et al.*, 2016). Fortunately, branching reactions are easy to identify such that a rational design approach might be able to achieve the same goals as ALE, e.g. genetic or protein engineering aiming at lower expression levels or affinities of branching enzymes could directly establish the desired growth phenotype.

Most efforts to establish synthetic C1 metabolism have so far focused on the RuMP cycle, the reductive glycine pathway, and the formolase pathway. Yet, given the diversity of metabolic solutions for C1 assimilation, more pathways are likely to be designed and tested, and the modular selection approach described here would be vital for their implementation. Currently, *E. coli* serves as the prime host for engineering synthetic C1 assimilation, mainly due to the highly developed genetic toolbox for its engineering and our extensive knowledge of its physiology and metabolism. However, other microbial hosts might prove to be more

suitable. Specifically, some microbes can produce specific chemicals better than *E. coli* and others display higher tolerance towards substrates or products. For example *Corynebacterium glutamicum* is arguably the best host to produce amino acids (Wendisch *et al.*, 2006), and *Saccharomyces cerevisiae* can tolerate high concentrations of the formate feedstock and alcohol products (Overkamp *et al.*, 2002; Mohd Azhar *et al.*, 2017). Also, some metabolic modules can directly integrate with C1 metabolism, making microbes that naturally harbour these better hosts. A primary example is the metal-dependent formate dehydrogenase, which can support highly efficient utilization of formate as cellular energy source but is difficult to introduce into a new host (Maia *et al.*, 2017).

In the next few years, we will likely witness the completion of synthetic C1 metabolism in model microbes. While we cannot predict which pathway will prove to be the most beneficial in the long term, it is clear that efforts such as those described in this review are bringing efficient bioproduction from C feedstocks closer.

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