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In Vivo Assimilation of One-Carbon via a Synthetic Reductive Glycine Pathway in *Escherichia coli*

Oren Yishai,[†] Madeleine Bouzon,^{‡,§,||} Volker Döring,^{‡,§,||} and Arren Bar-Even^{*,†}

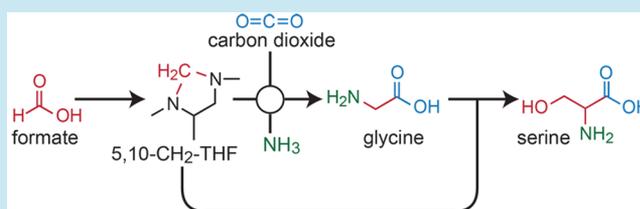
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ABSTRACT: Assimilation of one-carbon compounds presents a key biochemical challenge that limits their use as sustainable feedstocks for microbial growth and production. The reductive glycine pathway is a synthetic metabolic route that could provide an optimal way for the aerobic assimilation of reduced C1 compounds. Here, we show that a rational integration of native and foreign enzymes enables the tetrahydrofolate and glycine cleavage/synthase systems to operate in the reductive direction, such that *Escherichia coli* satisfies all of its glycine and serine requirements from the assimilation of formate and CO₂. Importantly, the biosynthesis of serine from formate and CO₂ does not lower the growth rate, indicating high flux that is able to provide 10% of cellular carbon. Our findings assert that the reductive glycine pathway could support highly efficient aerobic assimilation of C1-feedstocks.

KEYWORDS: formate metabolism, one-carbon metabolism, CO₂ fixation, auxotrophic strains, direct selection



As agricultural production of sugars has a limited capacity, their utilization for biotechnological purposes cannot be extended without severely undermining food security and decreasing biodiversity.^{1,2} For biorefineries to gain more ground, a sustainable feedstock for microbial growth must be identified.³ One-carbon (C1) compounds, such as methane,⁴ methanol,⁵ carbon monoxide,⁶ and formate,⁷ have been proposed as inexpensive and sustainable microbial feedstocks which could revolutionize bioproduction. However, aerobic assimilation of reduced C1 compounds into central metabolism is limited to three major routes in nature: the serine pathway, the ribulose monophosphate pathway, and the dihydroxyacetone pathway.⁸ These pathways are restricted to specialized groups of microorganisms and cannot be easily established in biotechnological hosts due to their metabolic complexity.^{9,10}

An effective way to tackle this problem is to design and implement tailor-made pathways that could potentially transform any microbe into an efficient C1-metabolizer.⁷ The reductive glycine pathway, shown in Figure 1, is a synthetic route suggested to support the most efficient aerobic assimilation of formate.^{7,10–12} The pathway consumes only two ATP molecules (and three NAD(P)H molecules) in the production of serine, which can be further metabolized to pyruvate or glycerate. As an additional benefit, the pathway assimilates ammonia, which could be transferred from serine to generate other amino acids, thus saving the ATP investment otherwise required for native ammonia assimilation. Since formate serves both as carbon and energy source (e.g., via formate dehydrogenase and oxidative phosphorylation), the high ATP-efficiency of the reductive glycine pathway translates to high efficiency of formate utilization, thus supporting higher biomass and product

yields than natural formate assimilation routes, for example, the serine pathway.⁸ Furthermore, its (relative) metabolic simplicity, restricted overlap with endogenous central metabolism, and ubiquitous occurrence of its enzymes, make the pathway a highly promising route for implementation in almost any microbe of biotechnological relevance.

In the reductive glycine pathway, formate is ligated to tetrahydrofolate (THF) and further reduced to 5,10-methylene-THF. The glycine cleavage/synthase system (GCS) reacts 5,10-methylene-THF with CO₂, ammonia, and NADH to generate the C2 amino acid glycine. Finally, glycine is condensed with another 5,10-methylene-THF molecule to produce the C3 amino acid serine. All reactions from formate to serine are fully reversible,^{13–15} and the overall thermodynamics of the pathway favor the reductive direction with $\Delta_r G^m \sim -6$ kJ/mol (change in Gibbs energy at pH 7.5, ionic strength of 0.25 M and reactant concentrations of 1 mM¹⁶). Hence, from a thermodynamic perspective, net production of serine is largely a matter of keeping the pathway substrates—formate and CO₂—at sufficiently high concentrations.

In a previous study, we demonstrated that, upon expression of the necessary enzymes in *Escherichia coli*, formate can serve as the sole precursor of activated C1 moieties.¹² These include 10-formyl-THF required for purine and histidine biosynthesis, 5,10-methylene-THF required for thymidine and coenzyme A biosynthesis, and 5-methyl-THF required for methionine and S-adenosyl methionine biosynthesis.¹⁷ In this case, externally provided glycine was condensed with a formate-derived 5,10-

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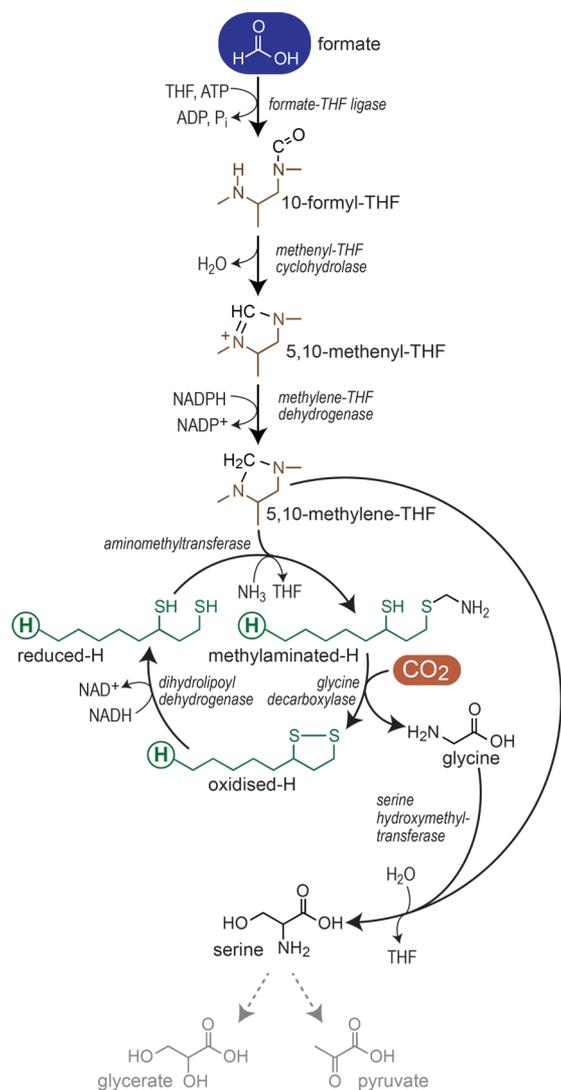


Figure 1. Metabolic structure of the reductive glycine pathway. The C1-carrying fragment of tetrahydrofolate (THF) is shown in brown. The H-protein of the glycine cleavage/synthase system, including the lipoic acid moiety, is shown in green. Enzyme names are written in italics.

methylene-THF to provide serine for cell growth.¹² This previous work, however, skipped the core segment of the reductive glycine pathway, that is, glycine biosynthesis from the reductive activity of the GCS.

The reductive activity of the THF enzymes and GCS is thought to have been the primordial route for the biosynthesis of glycine and serine.¹⁸ However, only a small group of anaerobic purine- and amino-acid-degrading microbes appear to retain the ability to produce glycine and serine from one carbon units.^{19–22} In most organisms the GCS and the THF system have reversed their activity, where serine and glycine—derived from central metabolism—are oxidized to generate activated C1 moieties.

Here, we construct tight selection schemes in which all possible natural routes for glycine or serine biosynthesis are blocked, thus generating truly auxotrophic strains. We show that a rational integration of native and foreign enzymes of the reductive glycine pathway enables an *E. coli* strain to satisfy all of its C1, glycine, and serine needs from formate and CO₂, together constituting ~10% of cellular carbon.²³ Our final strain, in which glycine and serine are produced from formate and CO₂, could grow at high growth rate (doubling time of ~1.7 h, reaching

maximal OD in 15 h), indicating that optimized expression can sustain high flux via the pathway. Together, our study provides a solid platform for further engineering of the reductive glycine pathway as a highly efficient route for the assimilation of C1 compounds.

RESULTS AND DISCUSSION

First, we aimed to achieve efficient conversion of formate and CO₂ to glycine. Toward this aim, we constructed an absolute glycine auxotroph strain, deleted in the genes encoding for serine hydroxymethyltransferase (Δ *glyA*), 2-amino-3-ketobutyrate CoA ligase (Δ *kbl*), threonine aldolase (Δ *ltaE*), and isocitrate lyase (Δ *aceA*, preventing production of glyoxylate which could potentially be aminated to glycine) (Figure 2a). This strain grows on a minimal medium only if supplemented with glycine. We transformed the strain with two plasmids, carrying both foreign enzymes as well as endogenous enzymes whose activity might be limiting. The first plasmid, pFD, carries the *Methylobacterium extorquens* AM1 gene encoding formate-THF ligase (Me-FTL, activity not native to *E. coli*) as well as the endogenous gene encoding the bifunctional 5,10-methenyl-THF cyclohydrolase/5,10-methylene-THF dehydrogenase (Ec-FoLD). The second plasmid, pTPH, harbors the endogenous genes encoding the proteins of the GCS: *gcvT*, *gcvP*, and *gcvH*. We expected that these plasmids would equip the cell with all necessary enzymatic activities to enable the biosynthesis of glycine from formate and CO₂ (Figure 2A). However, the transformed strain was unable to grow with 30 mM formate as a precursor for glycine biosynthesis, even at elevated CO₂ (10% CO₂, 90% air).

We reasoned that Ec-FoLD might be limiting the required reductive flux for glycine biosynthesis, as this enzyme is known to be inhibited by 10-formyl-THF²⁴ (the enzyme product in the oxidative direction). To replace this enzyme, we chose two enzymes from *M. extorquens*, 5,10-methenyl-THF cyclohydrolase (Me-Fch) and 5,10-methylene-THF dehydrogenase (Me-MtdA). As Me-Fch and Me-MtdA have evolved to carry high flux in the reductive direction (within the serine pathway),²⁵ we speculated that they would be able to support glycine biosynthesis at a sufficiently high rate to enable growth. Indeed, when we transformed the glycine auxotroph strain with the pTPH plasmid and a plasmid harboring the genes encoding Me-FTL, Me-Fch, and Me-MtdA (pFCM), we were able to select for growth on a minimal medium in which glycine is replaced with 30 mM formate (doubling time of 1.6 h; Figure 2b). This growth was not possible at ambient CO₂ concentration and could only be established at elevated CO₂ (10% CO₂, 90% air). The strict necessity for overexpressing the GCS enzymes indicates that their endogenous expression levels do not suffice to support high enough flux to supply all glycine and C1-units for cell growth. On the other hand, overexpression of lipoamide dehydrogenase (encoded by *lpd*)—a subunit of the GCS as well of pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase—was unnecessary.

To confirm the production of glycine from formate and CO₂, we conducted ¹³C-labeling experiments in which we fed the constructed strain with formate-¹³C, ¹³CO₂, or both. We measured the labeling of histidine (which contains a carbon derived from 10-formyl-THF), glycine, and serine. As expected, glycine was labeled once when formate-¹³C or ¹³CO₂ were used, and labeled twice when both were added to the medium (Figure 2c). Histidine was labeled once only when formate-¹³C was used and serine, generated from central metabolism, was unlabeled regardless of the feedstock (Figure 2c). The amino acid labeling

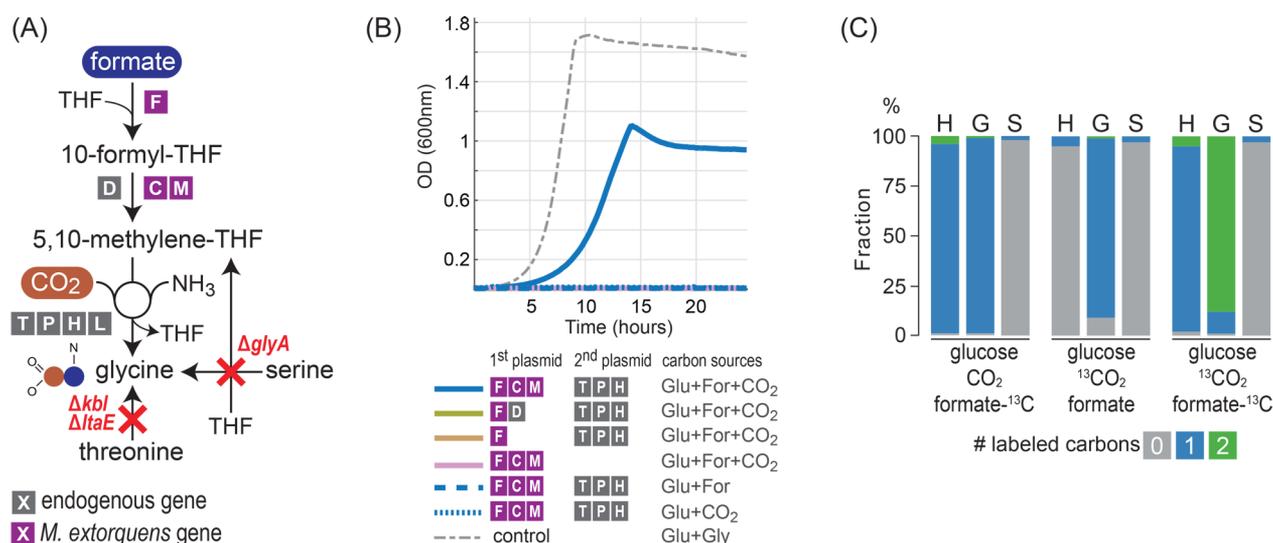


Figure 2. Biosynthesis of glycine and cellular C1-moieties from formate and CO₂. (A) Selection scheme for the production of glycine and cellular C1-moieties from formate and CO₂. *E. coli* enzymes are abbreviated within a gray background: (D) FolD, (T) GcvT, (P) GcvP, (H) GcvH, and (L) Lpd. *M. extorquens* enzymes are abbreviated within a magenta background: (F) FTL, (C) Fch, and (M) MtdA. (B) Biosynthesis of glycine and cellular C1-moieties from formate and CO₂ is possible only upon overexpression of FTL, Fch, and MtdA from *M. extorquens* and the endogenous GcvT, GcvP, and GcvH, and is possible only at high CO₂ concentration (10%). For this selection, the concentration of glucose was 10 mM and that of formate was 30 mM, resulting in a doubling time of 1.6 h. The control experiment used a strain without plasmids, and 1 mM glycine was added to the medium instead of formate. (C) ¹³C labeling experiments confirm that glycine and cellular C1-moieties are produced exclusively from formate and CO₂. The somewhat lower than expected labeling when ¹³CO₂ was used is attributed to the unlabeled CO₂ produced internally via glucose fermentation. Abbreviations: (H) histidine, (G) glycine, and (S) serine.

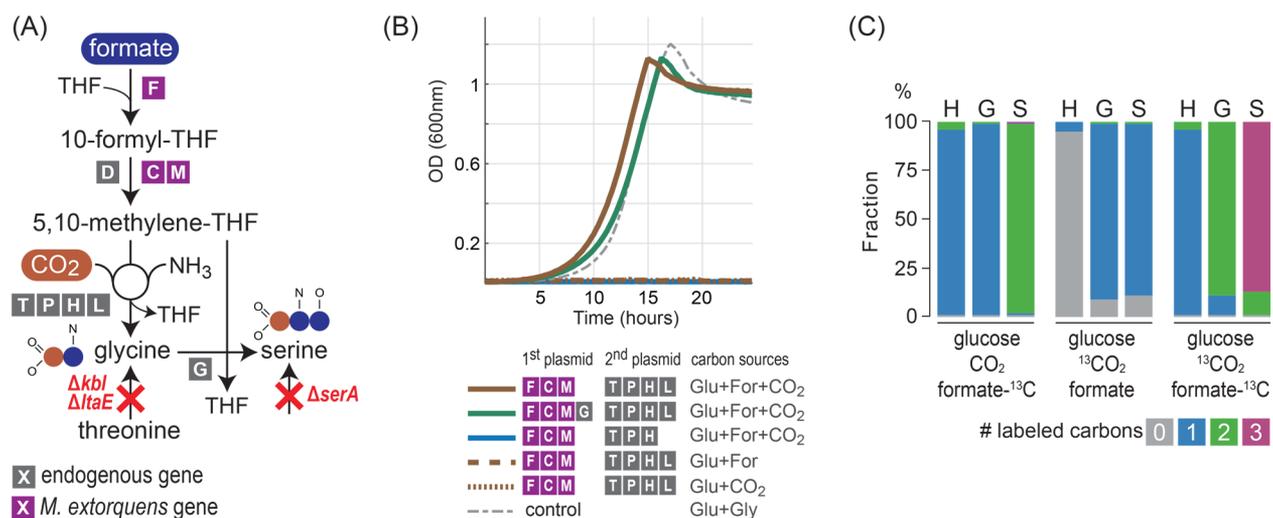


Figure 3. Biosynthesis of serine, glycine, and cellular C1-moieties from formate and CO₂. (A) A selection scheme for the production of serine, glycine, and cellular C1-moieties from formate and CO₂. *E. coli* enzymes are abbreviated within a gray background: (D) FolD, (T) GcvT, (P) GcvP, (H) GcvH, (L) Lpd, and (G) GlyA. *M. extorquens* enzymes are abbreviated within a magenta background: (F) FTL, (C) Fch, and (M) MtdA. (B) Biosynthesis of serine, glycine, and cellular C1-moieties from formate and CO₂ is possible only upon overexpression of FTL, Fch, and MtdA from *M. extorquens* and the endogenous GcvT, GcvP, GcvH, and Lpd, and is possible only at high CO₂ concentration (10%). For this selection, the concentration of glucose was 10 mM and that of formate was 30 mM, resulting in a doubling time of 1.7 h. The control experiment used a strain without plasmids, and 1 mM glycine was added to the medium instead of formate. (C) ¹³C labeling experiments confirm that serine, glycine, and cellular C1-moieties are produced exclusively from formate and CO₂. The somewhat lower than expected labeling when ¹³CO₂ was used is attributed to the unlabeled CO₂ produced internally via glucose fermentation. Abbreviations: (H) histidine, (G) glycine, and (S) serine.

thus confirms the production of glycine and C1-units *solely* from formate and CO₂.

Next, we aimed to demonstrate the production of serine from formate and CO₂ (unlike serine production from exogenously supplied glycine and formate, as we previously demonstrated¹²). Toward this goal, we constructed an absolute serine auxotroph strain, deleted in the genes encoding for 3-phosphoglycerate

dehydrogenase (Δ*serA*, first enzyme of serine biosynthesis pathway), 2-amino-3-ketobutyrate CoA ligase, threonine aldolase, and isocitrate lyase (Figure 3a). This strain could grow on a minimal medium only if supplemented with serine or glycine (the latter can be oxidized to C1 and converted to serine). Transformation of this strain with pFCM and pTPH did not support growth when serine/glycine was replaced with formate,

even at elevated CO₂. We speculated that the endogenous activity of a further pathway enzyme, which was not previously overexpressed, was limiting the high flux required for serine and glycine biosynthesis. This suggested two candidate genes for overexpression: *lpd* and *glyA*, the latter encoding serine hydroxymethyltransferase. We therefore constructed a plasmid harboring the endogenous *lpd* together with *gcvT*, *gcvP*, and *gcvH* (pTPHL). Transformation of the serine auxotroph with pFCM and pTPHL enabled growth with 30 mM formate and 10% CO₂ as sole precursors of glycine and serine (doubling time of 1.7 h; Figure 3b). The endogenous activity of serine hydroxymethyltransferase was sufficient to support maximal growth rate as overexpression of *glyA* did not further improve growth (Figure 3b).

To confirm the production of serine from formate and CO₂ we conducted ¹³C-labeling experiments in which we cultured the strain in the presence of formate-¹³C, ¹³CO₂, or both. We measured the labeling of histidine, glycine, and serine. As expected, serine was labeled once when ¹³CO₂ was used, twice when formate-¹³C was used, and three times when both formate-¹³C and ¹³CO₂ were added to the medium (Figure 3c). Histidine and glycine were labeled as in the experiments with the glycine auxotroph strain. The amino acid labeling thus unequivocally confirms the production of serine, glycine, and C1-units solely from formate and CO₂.

The findings presented in this study show that the biosynthesis of glycine and serine from the reductive activity of the THF system and the GCS can be established in a heterotrophic, aerobic bacterium. Notably, the biosynthesis of serine from formate and CO₂ did not change the doubling time, which remains ~1.7 h. This indicates that our optimized expression of pathway enzymes—using a strong constitutive promoter and a low copy number origin of replication for pFCM and a medium copy number origin for pTPHL (see Methods)—can support the required activity without limiting growth or imposing significant protein burden.

The high growth rate we achieved is in contrast to a recent study in which the reductive activity of the THF enzymes and GCS²⁶ resulted in slow growth (>50 h to reach maximal OD). Furthermore, this previous study observed low ¹³C-labeling of glycine and serine upon feeding with ¹³C-labeled formate and bicarbonate which clearly shows that these C1 feedstocks were not the main sources of the amino-acids. Instead, threonine cleavage probably supplied most of glycine and serine for the growth of these strains.²⁷

To establish growth on formate and CO₂ as sole carbon sources, the next step would include the overexpression of serine deaminase (or serine transaminase, hydroxypyruvate reductase, and glycerate kinase) as well as formate dehydrogenase to provide the cell with reducing power and energy. Moreover, to sustain such formatotrophic growth, the reductive flux via the THF enzymes and GCS will need to increase by an order of magnitude (providing 100% of biomass carbon instead of only 10%), which might further necessitate the modulation of cofactor levels (e.g., THF).

Despite the challenges associated with the establishment of the reductive glycine pathway, as nearly all microbial lineages harbor most of the components of the pathway, we suggest that it can be engineered as a plug-and-play metabolic module to enable C1 assimilation in almost any host of interest. We note that since methane and methanol can be oxidized to formate,⁸ these feedstocks might also be efficiently assimilated via this pathway.

The reductive acetyl-CoA pathway, the canonical anaerobic C1 assimilation route, supports a very high conversion efficiency of C1 substrates into a product of interest.²⁸ We put forward the reductive glycine pathway as the equivalent optimal metabolic solution for the aerobic assimilation of reduced one carbon compounds. In fact, the reductive acetyl-CoA pathway and the reductive glycine pathway share a structural similarity which points toward a generally efficient metabolic architecture: C1 is attached to THF and subsequently reduced and condensed, within a complex, with CO₂ to generate a C2 molecule (acetyl-CoA or glycine). The C2 molecule is then converted to a C3 molecule (pyruvate or serine) that can easily assimilate into the central metabolism, thus enabling microbial growth and production. Just as the reductive acetyl-CoA pathway can support autotrophic growth, the reductive glycine pathway could also be coupled with CO₂ reduction to enable autotrophic growth.²⁹ A recent paper suggests that this route could naturally support autotrophic growth in a phosphite oxidizing bacterium.³⁰ Altogether, the reductive glycine pathway could become a key metabolic route to enable efficient microbial growth and production using a wide range of sustainable feedstocks.

METHODS

Reagents. Primers were synthesized by Eurofins (Ebersberg, Germany). PCR reactions were performed using PrimeSTAR GXL DNA Polymerase (Clontech, Heidelberg, Germany). Restrictions were carried out using FastDigest enzymes, and ligations were performed using T4 DNA ligase and T4 polynucleotide kinase (T4 PNK), all purchased from Thermo Fisher Scientific (Dreieich, Germany). Glycine, potassium formate, and sodium formate-¹³C, were ordered from Sigma-Aldrich (Steinheim, Germany). D-Glucose was ordered from Carl Roth (Karlsruhe, Germany). ¹³CO₂ was ordered from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Synthetic-Operon Construction. Cloning procedures were designed using Geneious 8 (Biomatters, New Zealand). We cloned the genes encoding the following native *E. coli* enzymes (via PCR-amplification from *E. coli* MG1655 chromosomal DNA): serine hydroxymethyltransferase (coded by *glyA*), bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase (*folD*), aminomethyltransferase (*gcvT*), glycine decarboxylase (*gcvP*), H protein of the glycine cleavage system (*gcvH*), and lipoamide dehydrogenase (*lpd*). The genes originating from *Methylobacterium extorquens* AM1 were first codon optimized for *E. coli* K-12 (JCat³¹) and synthesized (Thermo Fisher). These genes encode for formate-tetrahydrofolate ligase (*ftfL*, UniProt Q83WS0), 5,10-methenyl-tetrahydrofolate cyclohydrolase (*fchA*, UniProt Q49135), and 5,10-methylene-tetrahydrofolate cyclohydrolase (*mtdA*, UniProt P55818). The genes were cloned into synthetic operons, where each gene is attached to ribosome binding site “C” (AAGTTAAGAGGCAAGA), using the BioBrick “no background” cloning technique.³²

The expression plasmids are based on the modular pZ plasmids pZA21MCS (p15A origin of replication) and pZS21MCS (pSC101 origin of replication).³³ The promoters of these plasmids were modified to have a constitutive, metabolically relevant expression level by using a mutated form of the *E. coli* phosphoglucose isomerase promoter (P_{pgi-mut}) that was reported to be >100 times stronger than the original (sequence of clone #10).³⁴ Preparation of the modified plasmids was done by PCR amplification of the original pZ plasmid with the pZpgi F and R primers, which contain the new promoter and

override the sequence of the old one (F- GTGGA ATTAT AGCAT TTTAG CCTTT AATTG TCAAT AGGTC TCGAG GTGAA GACGA AAGGG CCTCG, R- TCAGC AGGAC GCACT GACCG AATTC). The PCR reaction was incubated with DpnI, T4 PNK, and T4 DNA ligase, enabling the removal of template plasmid, and allowing circularization of the new plasmid, respectively. The kanamycin antibiotic resistance on the new plasmids were changed to streptomycin (Str^R) for the pSC101 based plasmid (and termed pZSSM), and tetracycline (Tet^R) for the p15A based plasmid (pZATM) allowing selection and coexpression of both plasmids in one strain. Synthetic operons harboring *ftfL*, *folD*, *fch*, *mtaA* with and without *glyA* were inserted into the pZSSM, and synthetic operons harboring *gcvT*, *gcvP*, and *gcvH*, with and without *lpd*, were inserted into the pZATM plasmid. Insertion of the synthetic operons to the plasmids described above was done by ligation, using the *EcoRI* and *PstI* restriction sites. A summary of strains and plasmids used in this study can be found in Table 1

Table 1

name	genotype	ref
Strains		
DH5 α	F ⁻ , λ^- , Φ 80 <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA</i> , <i>hsdR17</i> (r _K ⁻ m _K ⁺), <i>phoA</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Meselson et al. ³⁵
MG1655	F ⁻ λ^- <i>ilvG</i> ⁻ <i>rfb-50</i> <i>rph-1</i>	Blattner et al. ³⁶
glycine auxotroph	MG1655: Δ <i>glyA</i> , Δ <i>ttaE</i> , Δ <i>kbl</i> , Δ <i>aceA</i>	this study
serine auxotroph	MG1655: Δ <i>serA</i> , Δ <i>ttaE</i> , Δ <i>kbl</i> , Δ <i>aceA</i>	this study
Plasmids		
pZSSM	pSC101 ori; Str ^R ; P _{pgi} -mut	this study
pZATM	p15A ori; Tet ^R ; P _{pgi} -mut	this study
pF	pZSSM:: <i>ftfL</i>	this study
pFD	pZSSM:: <i>ftfL</i> , <i>folD</i>	this study
pFCM	pZSSM:: <i>ftfL</i> , <i>fch</i> , <i>mtaA</i>	this study
pFCMG	pZSSM:: <i>ftfL</i> , <i>fch</i> , <i>mtaA</i> , <i>glyA</i>	this study
pTPH	pZATM:: <i>gcvT</i> , <i>gcvP</i> , <i>gcvH</i>	this study
pTPHL	pZATM:: <i>gcvT</i> , <i>gcvP</i> , <i>gcvH</i> , <i>lpd</i>	this study

Bacterial Strains. *E. coli* strain DH5 α (F⁻, λ^- , Φ 80*lacZ* Δ M15, Δ (*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(r_K⁻ m_K⁺), *phoA*, *supE44*, *thi-1*, *gyrA96*, *relA1*) was used for all cloning procedures. *E. coli* strain MG1655 (F⁻ λ^- *ilvG*⁻ *rfb-50* *rph-1*) was used as the base strain for the auxotrophy strains presented in this study. Genomic gene deletions were performed using the Red/ET method,³⁷ recombining the selectable kanamycin resistance at the desired genetic locus (Quick & Easy *E. coli* Gene Deletion Kit, Gene Bridge, Heidelberg, Germany).

Growth Conditions. LB medium (1% NaCl, 0.5% yeast extract, and 1% tryptone) was used for strain propagation. Further cultivation was done in M9 minimal media (50 mM Na₂HPO₄, 20 mM KH₂PO₄, 1 mM NaCl, 20 mM NH₄Cl, 2 mM MgSO₄, and 100 μ M CaCl₂), supplemented with trace elements (134 μ M EDTA, 13 μ M FeCl₃·6H₂O, 6.2 μ M ZnCl₂, 0.76 μ M CuCl₂·2H₂O, 0.42 μ M CoCl₂·2H₂O, 1.62 μ M H₃BO₃, 0.081 μ M MnCl₂·4H₂O). Carbon sources were added according to strain and specific experiment: 10 mM glucose, 1 mM glycine, 30 mM formate, and/or 10% CO₂ (90% air).

Precultures were cultivated aerobically in 3 mL of M9 medium containing glucose and glycine, and were grown at 37 °C, shaking

at 220–250 rpm. Prior to inoculation in experimental conditions, cells were harvested by centrifugation (11 000 rpm, 30 s, 4 °C), washed three times in ice-cold M9, and inoculated in supplemented M9 media to an optical density of 0.005. Nunc 96-well microplates were used for growth experiments (Thermo Fisher Scientific), each well containing 150 μ L culture covered with 50 μ L mineral oil (Sigma-Aldrich) to avoid evaporation. (Note: the mineral oil cover enables gas transfer and thus supports fully aerobic growth.)

Growth experiments were carried out aerobically (either 100% air or 90% air, 10% CO₂) in a Tecan Infinite 200 Pro plate reader (Tecan, Switzerland) at 37 °C. Growth (OD₆₀₀) was measured after a kinetic cycle of 12 shaking steps, which alternated between linear and orbital (1 mm amplitude), and were each 60 s long. OD values measured in the plate reader were calibrated to represent OD values in standard cuvettes, which were found to correlate according to OD_{cuvette} = OD_{plate}/0.23. All growth experiments were performed in triplicate, and the growth curves shown represent the average of these triplicates (in all cases variability between triplicates was <5%).

Antibiotics were not used in the growth experiments, as plasmid retention is guaranteed via selection for the metabolic activities of the overexpressed enzymes.

Carbon Labeling. For stationary isotope tracing of proteinogenic amino acids,³⁸ cells were cultured in 3 mL of M9 media under conditions as described above, and were supplemented with either labeled or unlabeled carbon sources, for example, formate-¹³C and ¹³CO₂. For cultures grown in ¹³CO₂, a 10 L vacuum desiccator (Lab Companion) was used: after expelling the original gas content, the desiccator was refilled with 90% air and 10% ¹³CO₂. Following cultivation at 37 °C and after reaching the stationary phase, ~10⁹ cells were harvested by centrifugation for 1 min at 11 000 rpm. The biomass was hydrolyzed by incubation with 1 mL of 6N hydrochloric acid for a duration of 24 h in 95 °C. The acid was then evaporated by continued heating at 95 °C and streaming with air from above.

Hydrolyzed amino acids were separated using ultraperformance liquid chromatography (Acquity, Waters, Milford, MA, USA) using a C₁₈-reversed-phase column (Waters) as previously described.³⁹ Mass spectra were acquired using an Exactive mass spectrometer (Thermo Fisher). Data analysis was performed using Xcalibur (Thermo Fisher). Prior to analysis, amino-acid standards (Sigma-Aldrich) were analyzed under the same conditions in order to determine typical retention times.

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Notes

The authors declare no competing financial interest.

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